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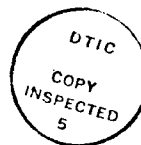
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DEVELOPMENT AND TESTING OF AN IN VITRO ASSAY FOR SCREENING  
OF POTENTIAL THERAPEUTIC AGENTS ACTIVE  
AGAINST Na CHANNEL NEUROTOXINS

ANNUAL/FINAL REPORT

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these studies, two agents have emerged as potential therapeutic agents; the compound HM-197 which is a part structure analog of tetrodotoxin, and a component from the venom of the scorpion *L. quinquestriatus* termed  $\beta$ -toxin. These agents alone have negligible functional effects on the sodium channel as assessed by electrophysiological measures, but are potent antagonists of the depolarizing neurotoxins batrachotoxin and veratridine (HM-197) and  $\alpha$ -scorpion toxins ( $\beta$ -toxin).

## SUMMARY

Based upon experimental observations of extensive conformational allostereism associated with ligand binding at the voltage-sensitive sodium channel, a rapid screening procedure has been developed to probe the interaction of new ligands/agents with a spectrum of binding domains on this vital mediator of electrical signalling in nerve and muscle. Under screening assay conditions, allosteric up- or down-modulation of specific batrachotoxinin-A benzoate binding is exploited as a sensitive indicator of the interactions of test substances with any of at least five different sodium channel domains. Interaction with the sodium channel protein or with sodium channel neurotoxins is a mechanistic prerequisite for any compound if it is to provide effective pharmacologic antagonism of sodium channel poisoning. The screening assay has therefore been reduced to practice and applied to the search for new sodium channel antitoxins or therapeutic compounds from several sources, including small molecular weight tetrodotoxin analogs, synthetic peptides, panels of monoclonal antibodies and polypeptide components of scorpion venoms. From these studies, two agents have emerged as potential therapeutic agents; the compound HM-197 which is a part structure analog of tetrodotoxin, and a component from the venom of the scorpion *L. quinquestriatus* termed  $\partial$ -toxin. These agents alone have negligible functional effects on the sodium channel as assessed by electrophysiological measures, but are potent antagonists of the depolarizing neurotoxins batrachotoxin and veratridine (HM-197) and  $\alpha$ -scorpion toxins ( $\partial$ -toxin).

## FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985)).

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## STATEMENT OF THE PROBLEM UNDER STUDY

As a primary mediator of the rising phase of the action potential in mammalian excitable cells, the voltage-sensitive sodium channel protein is vital to a variety of life processes. It is perhaps no too surprising, therefore, that this protein is a favored target for an impressively wide variety of naturally occurring neurotoxins elaborated by an equally wide variety of organisms for purposes of defense or predation. These sodium channel neurotoxins are among the most specific and potent toxins known. Specificity and toxicity are often associated with selective, high affinity binding interactions with the target, and these sodium channel neurotoxins have therefore been of fundamental importance as molecular probes in the study of the sodium channel protein structure and function(1,2).

Because of their value as research tools, the emphasis with regard to sodium channel neurotoxin research over the years has remained primarily on the search for new toxic compounds and analysis of their mechanism of action, preparatory to their further development as research tools. Consequently, relatively little attention has been paid to the search for potential antitoxins or therapeutic agents, even though several sodium channel neurotoxins present significant health hazards, e.g. toxic red tides in the Pacific North West and the Florida coast (*Gonyaulax catenella* saxitoxin and *Ptychodiscus brevis* brevetoxin, respectively) and scorpion envenomation (3,4,5). In part this lack of progress may be due to the fact that screening for such antitoxins requires the identification of agents that are not toxic themselves, and the various assays that have been so useful in the search for new toxins are simply not designed to detect "inactive" compounds.

In principle there are a number of possible approaches to the development of potential antitoxins and therapeutic strategies in general for protection against sodium channel neurotoxins. Structure-activity relationships within a known class of neurotoxins may provide clues to new structures that could serve as antagonists and lead to a program of synthesis. Our growing knowledge of the structure of the sodium channel protein itself may eventually provide information on the binding site topography for a particular neurotoxin and its relationship to channel function, permitting a rational synthetic design program for the development of antitoxins. Such knowledge could also form the basis for application of modern immunological techniques such as the production of specifically-acting monoclonal antibodies. In addition, the possibility should not be overlooked that Nature has already provided some useful, but as yet undiscovered, antitoxins.



Each of these approaches would be enhanced by the availability of a rapid screening assay that could detect interactions of test compounds at the sodium channel, whether these compounds arise from naturally-occurring sources, synthetic programs, or monoclonal antibody-producing myelomas. This central issue, the development of a rapid, reliable, and general screening procedure, has been a major focus in our current work. This report summarizes progress in the development of this assay and its reduction to practice in the identification of several potential antitoxins active at the voltage-sensitive sodium channel.

## BACKGROUND AND REVIEW OF EARLIER WORK

The voltage-sensitive sodium channel is perhaps the best understood example of the important general class of voltage-gated ion channels, membrane-spanning proteins that mediate ionic fluxes underlying the electrical activity of excitable cells in response to changes in transmembrane potential. Historically, this understanding has grown from elegant phenomenological descriptions of channel function based upon electrophysiological measurements. Even at this level naturally-occurring neurotoxins with specific effects on the voltage-sensitive sodium channel have been important tools, serving to perturb channel function in a variety of ways that facilitated the electrophysiological analysis of that function and mechanisms of action. In the last decade this research has entered a new and exciting phase with the purification, characterization and sequencing of the sodium channel protein(s) from several sources (6,7,8). In this new arena, specifically acting neurotoxins take on an increasingly important role by providing the tools necessary to probe discrete regions of the channel structure that serve as neurotoxin binding sites. Among all voltage-gated channels, the sodium channel enjoys a distinct advantage here as a target for study because of the impressively large number of naturally-occurring neurotoxins that act specifically and with high affinity at different sites on this channel protein. These toxins include, but are not limited to, the channel blockers tetrodotoxin (TTX) and saxitoxin (STX), the channel activators batrachotoxin (BTX), veratridine and aconitine, the polypeptide  $\alpha$ -toxins from scorpion and sea anemone that slow the process of channel inactivation in contrast to the scorpion  $\beta$ -toxins that affect channel activation, and the polyether brevetoxins isolated from the dinoflagellate *Ptychodiscus brevis*. Each of these toxin groups is known to have a different binding site on the sodium channel. In the context of the current work we have focussed on the steroidal alkaloid batrachotoxin, the prototypical and most potent member of the so-called "lipid soluble" group of channel activator neurotoxins. One of the most interesting aspects of the action of BTX and its congeners is that every functional property of the sodium channel is altered upon binding to a single site (reviewed in 9). BTX is known to simultaneously shift the voltage dependence for channel activation to more negative membrane potentials, block the inactivation process and associated gating charge movement, and increase the apparent physical dimensions of the selectivity filter with concomitant changes in channel conductance and relative ion permeability. This observation suggests that the BTX binding site lies in a key region of the sodium channel that is connected to other domains through significant conformational dynamics underlying channel function.

This general theme has been supported by work both from this laboratory and others in which the allosteric interactions of a variety of sodium channel neurotoxins and ligands with the batrachotoxin binding site have been documented with direct radioligand binding measurements utilizing the biologically active probe [ $^3\text{H}$ ]batrachotoxinin-A benzoate (BTX-B) (10). These results are summarized in Table I and have been recently reviewed (9).

A detailed protocol for the screening procedure is included as an appendix to this report (A rapid screening procedure for the detection of compounds active at the voltage-sensitive sodium channel: A manual). Briefly, the procedure is based on the standard [ $^3\text{H}$ ]BTX-B binding assay, but binding is equilibrated in the presence of several unlabeled effectors, including TTX, the pyrethroid deltamethrin, and *L. quinquestriatus* scorpion venom, each at approximately half-maximal concentration. Under these conditions, the specific binding of BTX-B is delicately balanced by the influence of both allosteric inhibition and enhancement, and is poised to respond sensitively to changes in that balance induced by test agents acting at any of at least five distinct channel sites. Active agents are identified either by an observed increase or decrease in specific BTX-B binding. The assay has the utility of casting a rather broad net in a single procedure and hence can be quite useful in a variety of circumstances, such as the screening of a panel of monoclonal antibodies raised against channel epitopes, the testing of chromatographic fractions of toxic venoms, or the screening of potential therapeutic compounds resulting from a program of synthesis. Once a test compound has been flagged as being active on the sodium channel, however, further biochemical and/or electrophysiological tests are required to determine the site and mechanism of action. In our work we have utilized both electrophysiological measures and direct binding studies with [ $^3\text{H}$ ]STX to further characterize potentially active compounds.

## RATIONALE AND APPROACH TO THE PROBLEM

In order to develop a practical screening assay procedure and then reduce it to practice, the work has proceeded through two distinct phases. In the first phase, a number of variables concerning the screening assay conditions were optimized. These included i) concentrations of labeled BTX-B and unlabeled effectors required to provide a sensitive response, and ii) preparation of synaptoneurosomal membrane fractions from rat cerebral cortices and optimization of conditions to permit frozen storage over extended periods of time without deterioration of membrane potential or other factors that would adversely affect use in the screening assay following thawing and resuspension. Following optimization of these factors and conditions, the assay was subjected to a validation process in which a number of well-known and characterized sodium channel-active agents were "screened" in order to verify that the assay responded correctly in accord with the known properties of the test compounds.

In phase two of the work, the assay was applied as a routine screening procedure to identify potential new antitoxins or therapeutic agents. The rationale for use of the assay in this regard is based on the premise that antitoxins or agents with therapeutic potential must from a mechanistic standpoint act either at the sodium channel protein or with the offending toxin itself in order to be effective. Since the screening assay conceptually can monitor binding interactions at at least five, and possibly more, different sodium channel domains (i.e., those to which the BTX binding site is allosterically coupled), and in addition, includes four classes of sodium channel ligands present in the assay solution, both mechanistic modalities can be broadly screened in a single procedure. The initial experiments were focussed on several small molecular weight compounds that were suspected of potentially exerting antitoxic effects based on earlier work or literature reports. One of these compounds, CGA-98496, a cypermethrin stereoisomer and a representative of pyrethroid type insecticides, was found to be a potent antagonist of [ $^3\text{H}$ ]BTX-B binding in synaptoneurosomes (15). However, CGA-98496 is 30,000 times less potent than the most active stereoisomer in insect bioassays (20), presumably reflecting a relative lack of functional effects on the sodium channel. Two other compounds, benzimidazole and 5-benzoyloxy-2-imino-hexahydropyrimidine (HM-197), were also of interest as potential antitoxins. These compounds have been investigated for their effects as TTX and STX analogs in blocking compound action potentials in frog sciatic nerve. Both were found to produce at least partial blockade, but relatively high concentrations of 10 and 1 mM, respectively, were required (21,22). However, we have found that HM-197 at a

concentration of 3  $\mu$ M produced 25% inhibition of BTX-B binding, presumably by the same mechanism as TTX or STX-induced inhibition (16). We therefore considered the possibility that these analogs might bind with relatively high affinity to the sodium channel at the TTX site yet have low efficacy as a channel blocker, thus serving as non-toxic blockers of TTX/STX binding.

A second focus for application of the screening assay was the polypeptide scorpion sodium channel neurotoxins. More than 100 such polypeptides have been purified from various scorpion species to date. The venom of a single species is a complex mixture of as many as 20-30 different components, among which may be several isoforms of  $\alpha$ - and/or  $\beta$ -neurotoxins. The isoforms of each group are characterized by extensive sequence homologies, even between different species of scorpion, and similar modes of action, whereas the slight differences in amino acid sequence may be reflected in different potencies or target species specificities. For example, a particular  $\alpha$ -toxin may be more potent in insects than in mammals. ( See ref. 233 for a recent review). While progress in this area of research has been remarkable, one might adopt a different perspective and note that the majority of scorpion venom components have been ignored since they are not toxic. We have been interested in the possibility that an isoform or isoforms might exist among these unstudied components that, because of critical sequence differences, could still bind the sodium channel yet lack efficacy as a toxin; that is, components that might be useful as antitoxins. Such components, if present, would be expected to be found at relatively low concentrations in toxic venoms where the antitoxin effects are overridden by the neurotoxins present at higher concentrations. In order to investigate this possibility we have therefore adopted the strategy of fractionating the venom by HPLC and testing the resulting fractions by the screening assay which is well-suited to the purpose.

A second strategy to explore potential scorpion toxin antitoxins involved the use of the screening assay to evaluate panels of monoclonal antibodies raised against synthetic sodium channel peptides. By comparing the amino acid sequences of ligand and receptor site pairs in a series of examples for which such sequence information is available, such as the interleukin II receptor system and the  $\alpha$ -bungarotoxin/acetylcholine receptor system, Dr. D. Dwyer, a colleague, made the empirical observation that for a given pair, receptor and ligand share discrete regions of significant sequence homology (Dwyer et al., unpublished observations). In extending this analysis to  $\alpha$ -scorpion toxin interaction with the sodium channel, we found a region of the sodium channel sequence that shares significant homology with the amino-terminal portion of  $\alpha$ -scorpion toxins such as toxin V

from *Leiurus quinquestriatus*. Interestingly, this sodium channel sequence, amino acids 1509 - 1536 as defined by Numa and colleagues (24), is in that region of the channel postulated to be involved in the process of inactivation, i.e. that process which is affected by the binding of  $\alpha$ -scorpion toxins (233) and is one of the most highly conserved regions of the protein across isoforms from rat brain and eel electroplax sodium channels (25). Given the sequence homology of the synthetic peptide with scorpion toxins and the possibility that the corresponding portion of the sodium channel protein could be involved with the scorpion toxin binding site, several possibilities emerge for activity of the antibodies in the screening assay; i) the antibody might bind at the scorpion toxin site and mimic the effect of scorpion toxin, leading to an increase in BTX-B binding, ii) the antibody might bind this site and block the effect of scorpion toxin, leading to a decrease in BTX-B binding, or iii) the antibody might bind the scorpion toxin, also resulting in decreased BTX-B binding. To test these hypotheses, we prepared monoclonal antibodies directed against the appropriate synthetic sodium channel peptide and evaluated their activities with the screening assay.

## EXPERIMENTAL METHODS

A. Buffers. HEPES buffer was composed of 130 mM choline chloride, 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 5.5 mM glucose, 0.8 mM  $\text{MgSO}_4$ , and 5.4 mM KCl, adjusted to pH 7.4 with Trizma base. WASH buffer was composed of 163 mM choline chloride, 5mM HEPES, 1.8 mM  $\text{CaCl}_2$ , and 0.8 mM  $\text{MgSO}_4$  adjusted to pH 7.4 with Trizma base. TES buffer consisted of 20 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 112 mM NaCl, 2.5 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 1.3 mM  $\text{CaCl}_2$ , 15 mM  $\text{NaHCO}_3$ , 0.4 mM  $\text{KH}_2\text{PO}_4$ , and 10 mM glucose adjusted to pH 7.4 with NaOH. Modified TYRODE'S solution contained 135 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{Na}_2\text{HPO}_4$ , 15 mM  $\text{NaHCO}_3$ , and 11 mM glucose, pH 7.4. Isotonic SUCROSE solution consisted of 0.32 M sucrose, 10 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.4.

B. Synaptoneurosomes. Synaptoneurosomes were prepared by a modification of the procedure described by Creveling et al. (12). Briefly, freshly dissected cerebral cortical tissue from male Sprague-Dawley rats was homogenized in 2 volumes (wt/vol) of ice cold HEPES buffer using 10 strokes of a loose-fitting glass-glass homogenizer. The homogenate was diluted with 2 additional volumes of cold buffer and centrifuged at 1000xg for 15 min at 5°C. The supernatant was discarded and the pellet was resuspended in HEPES buffer ( or other buffers as indicated) by repetitive pipetting with a 9 inch Pasteur pipette or by gentle homogenization in a loose-fitting glass-glass homogenizer. This preparations was used directly as "unfiltered synaptoneurosomes". For preparation of "filtered synaptoneurosomes", the pellet from the 1000xg centrifugation was resuspended in 20 volumes of HEPES buffer (original wt/vol) by homogenization using 3 strokes of a loose-fitting glass-glass homogenizer and filtered through a Millipore AP prefilter overlaid with 3 layers of HC-3 160 mesh nylon (Tetko, New York). The filtrate was collected in ice cold tubes and subsequently filtered through a Millipore LCWP 047 filter having a 10  $\mu\text{m}$  cut-off. This filtrate was then centrifuged at 1000xg for 30 min at 5°C, and the pellet resuspended in buffers of choice for use. Protein concentration for both filtered and unfiltered preparations was generally adjusted to approximately 6 mg/ml. Protein determinations were performed using the procedure of Peterson (26) with bovine serum albumin as a standard. Alternatively, protein concentration was estimated by dilution of 75  $\mu\text{l}$  of the tissue suspension in 1.5 ml distilled water and recording the absorbance at 280 nm (uncorrected for light scattering). The absorbance reading was converted to protein

concentration with reference to a standard curve calibrated against protein concentrations determined with the Peterson procedure.

C. Measurement of tritiated toxin binding. Measurement of specific [ $^3\text{H}$ ]BTX-B binding was performed as reported previously (16). Briefly, standard binding reactions were initiated by addition of 150  $\mu\text{l}$  of synaptoneurosomes suspension in HEPES buffer containing approximately 1 mg protein to a solution in HEPES buffer of [ $^3\text{H}$ ]BTX-B and various concentrations of unlabeled effectors as indicated. The concentration of labeled toxin was generally 8-9 nM, and the total assay volume was 320  $\mu\text{l}$ . The effectors were added from concentrated stock solutions (*L. quinquestriatus* scorpion venom, 2 mg/ml water; TTX, 100  $\mu\text{M}$  in water; veratridine, 10 mM in MeOH; pyrethroids, 5 mM in MeOH; all others as indicated). For the standard screening assay, the concentrations of TTX, deltamethrin, and scorpion venom in the assay were 25 nM, 1  $\mu\text{M}$ , and 2.5  $\mu\text{g/ml}$ , respectively. Incubations were carried out for 45 min at room temperature and were then terminated by addition of 3 ml cold WASH buffer. The tissue was immediately collected on Whatman GF/C glass fiber filters using a Brandel 30-place filtration manifold, and washed 3 more times with 3 ml cold WASH buffer. Radioactivity associated with the tissue was determined by liquid scintillation spectroscopy of the filters suspended in 10 ml scintillation cocktail (3a70B, RPI). Nonspecific binding was determined from parallel assays containing 250  $\mu\text{M}$  veratridine and has been subtracted from the data. For further details of the standard screening assay, see the Appendix.

For the determination of [ $^3\text{H}$ ]BTX-B dissociation rates, synaptoneurosomes were pre-equilibrated with 9 nM labeled toxin and 12.5  $\mu\text{g/ml}$  *L. quinquestriatus* scorpion venom with and without test agent in parallel. Following the pre-equilibration period, at time =  $t_0$ , excess veratridine was added (300  $\mu\text{M}$ ) to all tubes. At various time points over the next two hours, triplicate samples from assays both with and without test agent were filtered and the specific binding determined by liquid scintillation spectroscopy as above.

Data points for all binding experiments were determined in triplicate and are presented as the mean  $\pm$  S.D. of these determinations. Unless otherwise indicated, figures show the results of 1 experiment that is representative of 2 or more separate determinations.

D. Fluorescent measurement of relative membrane potential. Freshly prepared synaptoneurosomes and synaptoneurosomes arising from various treatments (frozen storage) were diluted into TES buffer to a concentration of approximately 3 mg protein/ml.



One hundred microliters of these suspensions were added to cuvettes containing 1.8 ml of a solution of 3-3'-diethyloxadicarbocyanine (80  $\mu$ M) in TES buffer and allowed to equilibrate while monitoring fluorescence at 600 nm (580 nm excitation). After a stable baseline had been achieved (approximately 3 min), 50  $\mu$ l of a 4 M KCl solution was added to give a concentration of 102 mM, leading to depolarization of the synaptoneurosomes. The resulting increase in fluorescence intensity,  $\Delta F$ , was recorded for fresh synaptoneurosomes and test samples. The relative membrane potential of the test samples was then expressed as a percentage of that of the fresh synaptoneurosomes by the expression  $(\Delta F_t/\Delta F_f) \times 100$ . Previous work has shown that the fluorescence response of the dye is a linear function of membrane potential in this system (27).

E. Electrophysiological measurements. The diaphragm and phrenic nerve were dissected from 150 g rats under pentobarbital anesthesia and a 5-10 mm diameter strip of muscle with nerve attached was suspended in an apparatus permitting measurement of the nerve compound action potential. The apparatus is a modification of that described earlier by Pagala (28). The muscle was fixed at the bottom of a glass chamber and connected to a force transducer at the top to measure muscle tension. Two pairs of platinum electrodes contacted the phrenic nerve for stimulation and recording of the nerve compound action potential. The muscle could be stimulated directly *via* two plate electrodes and the muscle compound action potential recorder by another platinum electrode situated just below the top tendon. (See Figure 1). The entire arrangement was immersed in TYRODE'S solution and bubbled continually with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The apparatus was surrounded by a thermostatted circulating water jacket and the temperature controlled at 30°C. In order to collect the data presented here, the solution level in the apparatus was lowered below the level of nerve insertion in the muscle, and the distal nerve electrodes used to deliver supramaximal stimulating square wave pulses of 0.1 msec duration at a frequency of 100 Hz for 1 sec unless otherwise indicated. The resulting train of nerve compound action potential recorded at the proximal pair of electrodes was displayed on a storage oscilloscope and photographed with Polaroid film to produce a permanent record. Following these brief recording periods, the fluid level in the bath was restored to full height. Control experiments have demonstrated that, under these conditions, reproducible responses of the preparation may be obtained over a period of 5-6 hours.

F. Scorpion venom fractionation. *L. quinquestratus* scorpion venom (Sigma Chemical Co., St. Louis) was dissolved in 0.1 M ammonium acetate, pH 6.9/15% isopropanol at a concentration of 4-40 mg/ml by stirring at 5°C for one hour. The mixture was then

centrifuged at 100,000 x g at 5°C for one hour to pellet the insoluble mucopolysaccharides. The clear supernatant was harvested and passed through a Rainin LX-N66045 pre-filter prior to storage at -70°C or fractionation by HPLC. Reverse phase gradient elution HPLC (solvent A = 0.1 M ammonium acetate, pH 6.9 with a conductance of 12.9 mSiemens and solvent B = isopropanol ) over a Dynamax C18 column (Rainin Instrument Co.) was performed on a Rainin instrument consisting of two HP-Rabbit pumps, pressure monitor, mixing chamber, Rheodyne injection manifold with either a 20 µL (analytical scale) or 100 µL (preparative scale) sample loop. The system was interfaced to an Apple computer for control of flow rates and gradient production. Column effluent was monitored with an ISCO V<sup>4</sup> variable absorbance monitor set at 254 nm and led to a Gilson model 201 programmable fraction collector. Fractions were originally concentrated for further use by rotary evaporation in a 40°C water bath to strip the isopropanol followed by lyophilization to remove water and the ammonium acetate. This procedure was subsequently found to give unreliable recovery of fractions of interest and the method for concentration and drying of fractions was therefore changed to a Savant Speed-Vac procedure. The proteinaceous residues were generally redissolved in a minimal volume of 0.1 M ammonium acetate, pH 6.9, for further use or storage at -20°C. Additional HPLC purification of fractions of interest was carried out on an Aquapore BU300 column (2.1 x 100 mm) using a gradient of solvent A (10% isopropanol in 0.1% trifluoroacetic acid) and solvent B (70% isopropanol in 0.1% trifluoroacetic acid). The effluent was monitored by UV absorption at 280 nm.

G. Electrophoresis and Western blotting. Scorpion toxin fractions were subjected to SDS gel electrophoresis according to the Laemmli procedure (29) using a Hoeffer Mini-Gel apparatus and a 15% gel. Native gel electrophoresis was carried out as described by Catterall (30). SDS gels were blotted onto nitrocellulose membranes using the Poly-Blot apparatus from American Bionetics, Inc., according to the manufacturer's instructions. Blotted proteins were probed with antibodies using the procedures of Birk and Koepsell (31) to minimize nonspecific antibody binding and visualized with an HRP-coupled second antibody developed with diaminobenzidine as a substrate (32 ).

## RESULTS

### A. Development and Validation of the Screening Assay

Details of assay development and validation experiments are contained in the previous Annual Reports. In addition, a manual providing complete information on all aspects of the screening assay protocol has been prepared and is supplied as an appendix to this report (A rapid screening procedure for the detection of compounds active at the voltage-sensitive sodium channel: A manual).

One of the objectives in the development of the screening assay was to insure that the protocol was accessible to moderately equipped laboratories and suitable for routine use. For this reason, the synaptoneurosomal membrane preparation from rat cerebral cortex was selected as the source of sodium channel protein instead of the more traditional synaptosome preparation. Synaptoneurosomes behave similarly to synaptosomes in all relevant aspects related to binding of sodium channel ligands, but can be prepared in approximately 30 min in contrast to the 3-4 hr required for preparation of synaptosomes (33). In order to further simplify the protocol, it would obviously be useful if the tissue preparations could be prepared in advance, stored in a suitable manner, and retrieved when required for use in the assay. A number of variables such as tissue handling, buffers, protease inhibitors, thawing conditions etc., were assessed for their effect on the stability of synaptoneurosomal preparations to long term frozen storage at  $-70^{\circ}\text{C}$ . Figures 2 and 3 show the major result that synaptoneurosomal preparations remain viable for use in the screening assay for up to four months when stored frozen at  $-70^{\circ}\text{C}$  in 0.32M sucrose. After this time, 70-80% of the original specific binding activity of [ $^3\text{H}$ ]BTX-B remains. Longer storage results in continuing degradation of toxin binding and is therefore not recommended. The integrity of the TTX/STX site in frozen synaptoneurosomes was also tested. As shown in Figure 4, TTX inhibits binding with an apparent dissociation constant of 17 nM, in good agreement with previous results (16). This experiment demonstrates that this assay can be used directly as an adjunct to the standard screening assay in order to test for interactions at the TTX/STX site specifically without altering requirements for preparation or storage of the tissue.

The intent of the screening assay is to provide a method to detect interaction of test compounds with the sodium channel in a manner that would be both sensitive and broad in scope. The allosteric interactions associated with BTX-B binding confer upon this measure the ability to monitor interactions at multiple sodium channel domains, thus providing a

broad scope. In order to maximize the sensitivity and response of BTX-B binding to test agents, complete dose-response curves for the effects of TTX, deltamethrin, and  $\alpha$ -scorpion toxin alone on specific [ $^3$ H]BTX-B binding were examined. The curves were then repeated for each unlabeled effector in the presence of half-maximal concentrations of the other effectors as determined from the first set of experiments. Based on these results, the following concentrations of the unlabeled effectors were selected for inclusion in the standard screening assay: TTX, 25 nM; deltamethrin, 1  $\mu$ M; scorpion toxin, 2.5  $\mu$ g/ml of the lyophilized venom of *L. quinquestriatus*. These concentrations were selected to produce approximately half-maximal effects mediated through these different sodium channel sites in the assay. Thus, the binding of [ $^3$ H]BTX-B should be highly sensitive to allosteric modulation induced by either agonist or antagonist properties of test compounds acting at these sites. In the absence of any test compounds and in the presence of unlabeled toxins at the indicated concentrations, equilibrium binding of 9 nM [ $^3$ H]BTX-B to synaptoneurosomes at a concentration of 3 mg protein/ml yields approximately 4000 dpm of total binding, of which 50% is specific.

Once conditions for the screening assay were finalized, validity was tested by examining the effects of twelve different compounds on the binding of BTX-B. The list of compounds was selected to include compounds that were known to act at five different sodium channel binding domains and whose effects could be predicted, as well as several compounds whose activity at the sodium channel was unknown. The results are shown in Table II. The response to each of the sodium channel-active agents was as predicted, showing that under standard screening assay conditions this measurement reports faithfully on ligand-channel interactions at at least five sites, i.e. BTX, TTX/STX, local anesthetic, pyrethroid, and  $\alpha$ -scorpion toxin binding domains.

#### B. HM-197 as a Potential Therapeutic Agent

As mentioned previously, 5-benzoyloxy-2-imino-hexahydropyrimidine or HM-197 was found to significantly inhibit the binding of BTX-B with a  $K_{0.5}$  of 35  $\mu$ M under standard screening assay conditions, whereas much higher concentrations were reported to be necessary for any effect on action potentials. To test the hypothesis that HM-197 might act as an antagonist of toxin binding at the TTX/STX site, complete dose response curves for inhibition of both STX and BTX-B binding in the absence of other effectors was obtained. These results are presented graphically in Figure 5, showing that HM-197 has little effect on STX binding even at concentrations as high as 400  $\mu$ M, while the half-maximal concentration for inhibition of BTX-B binding is 35  $\mu$ M. Thus the effect of HM-

197 is not mediated through an interaction at the STX/TTX binding site, an observation that was somewhat surprising in view of the fact that HM-197 was originally prepared as a structural analog of the channel blockers (21).

Additional experiments were carried out to determine if HM-197 inhibits BTX-B binding by a direct effect on the BTX-B binding site. If inhibition were due to simple competitive antagonism at that site, the off-binding rate for [ $^3\text{H}$ ]BTX-B should not be altered by the presence of HM-197. The result shown in Figure 6 indicates that HM-197 decreases the half time for BTX-B dissociation by a factor of 1.6 and that inhibition therefore must occur by an allosteric mechanism mediated by binding of HM-197 at a site other than the BTX-B binding site.

Using an electrophysiological approach described in previous Annual Reports, we also tested the hypothesis that HM-197 acts like a local anesthetic to inhibit binding of BTX-B. The experiment shown in Figure 7 demonstrates that this was not the case. Control responses of a rat phrenic nerve mounted in the chamber described in Experimental Methods are shown in panel A. The nerve was stimulated supramaximally at 100 Hz (0.5 msec pulse width) for 1 second and the resulting 100 action potentials superposed on a storage oscilloscope. This control response was unaltered by pre-equilibration with 100  $\mu\text{M}$  HM-197, demonstrating that this compound does not affect the functional aspects of the sodium channel at concentrations where the binding of BTX-B is strongly inhibited. The effects of nesacaine (2-chloroprocaine hydrochloride) at a concentration of 150  $\mu\text{M}$  following 30 min pre-incubation are presented in panel B. Panel C shows that pre-equilibration of the nerve with 100  $\mu\text{M}$  HM-197 prior to addition of nesacaine did not appreciably alter the response to the local anesthetic, suggesting that HM-197 does not bind to the local anesthetic binding domain.

Using the same electrophysiological paradigm, we tested the ability of HM-197 to protect against the actions of veratridine, a depolarizing neurotoxin acting at the same site as BTX. Pre-equilibration of the nerve with 7  $\mu\text{M}$  veratridine abolished the action potential as expected. Subsequent addition of HM-197 failed to reverse the depolarization-induced action potential block. However, co-administration of these agents did reveal significant protection by HM-197. This result is shown in Figure 8. Figure 8 A presents the control response of the nerve to the stimulus train. The response after 60 min incubation with 7  $\mu\text{M}$  veratridine alone is shown in Figure 8 B, and the response after 60 min co-incubation with 7  $\mu\text{M}$  veratridine and 50  $\mu\text{M}$  HM-197 is demonstrated in Figure 8 C. These results

corroborate the findings from the binding studies, and indicate that at concentrations where it alone has no detrimental effects, HM-197 may provide protection against the depolarizing effects of toxins acting at site 2 of the sodium channel.

### C. Scorpion neurotoxins

Previous Annual Reports have described two approaches to investigate potential antitoxins or therapeutic agents directed against  $\alpha$ -scorpion toxins such as toxin V from *L. quinquestriatus*. Both of these approaches have relied on the screening assay. In one case, panels of monoclonal antibodies directed to sodium channel sequences sharing homology with N-terminal regions of scorpion toxin neuropeptides were prepared and examined for effects on the sodium channel under standard screening assay conditions. A typical result is shown in Figure 9. In general there were no robust effects of these monoclonal antibodies detected in the screening assay, although an indication of potential inhibitory effects was noted for monoclonals PB 6,7, and 9. More robust effects might require higher concentrations of the monoclonals, since the experiments we carried out used the relatively dilute supernatant solutions from hybridoma cultures directly that were then further diluted into the assay mixture. The lack of effect of PB 29 was somewhat surprising, since this monoclonal antibody was shown to cross-react with toxic *L. quinquestriatus* components in Western blots. It is possible that the scorpion toxin epitope recognized by the antibody is not involved in the binding interaction with the sodium channel protein.

In a related group of experiments, another synthetic sodium channel peptide segment was studied. This peptide bears sequence homology to *Androctonus australis* (Hector) scorpion neurotoxin, again in the N-terminal region, as shown in Figure 10. We hypothesized that such homologous sequences may provide some of the interactions of binding (e.g. "like binds like") between ligand and the sodium channel. Thus, a synthetic peptide could conceivably function as a soluble "pseudoreceptor" and prevent ligand interaction at the sodium channel by serving as a false receptor site. Alternatively, if the shared sequence is involved in a functionally significant conformational change in the channel structure, for example by mediating the docking of adjacent protein segments, then the appropriate synthetic peptide might in fact mimic the action of neurotoxin. To test this hypothesis, the effects of this synthetic peptide (NAaHI) were examined using the screening assay. Original reports of these experiments in the Quarterly Report of April 7, 1989, showed that NAaHI inhibited [ $^3$ H]BTX-B binding at very low concentrations. Subsequent studies, however, revealed that this effect was artifactual due to progressive

loss of deltamethrin as a function of time in the serial dilutions of peptide NAaHI. Deltamethrin is very hydrophobic and will partition out of aqueous assay buffers unless the synaptoneurosomal brain tissue is present when deltamethrin is added. In the initial NAaHI experiments, serial dilutions of the peptide were prepared in the screening assay toxin cocktail and aliquots of the dilutions were subsequently added to synaptoneurosome suspensions. The time-dependent loss of deltamethrin occurring in the pre-prepared serial dilutions while aliquots were being pipetted to assay tubes (lowest concentration added last) led to the apparent dose-response relationship for inhibitory effects of NAaHI. When this artifact was recognized and corrected, no effects of NAaHI up to a concentration of 50  $\mu$ M could be discerned.

In order to investigate the hypothesis that toxic scorpion venoms contain components, albeit at low concentrations, that have antagonist properties (i.e. antitoxins), *L. quinquestriatus* scorpion venom was fractionated by HPLC and individual fractions tested with the screening assay. Following optimization of HPLC conditions as detailed in previous Annual Reports, a standard protocol was adopted for the initial purification of antitoxin venom components as shown in Figure 11. Briefly, venom was dissolved in 100 mM ammonium acetate ( $\text{NH}_4\text{OAc}$ ). Aliquots were chromatographed on a Dynamax C18 column using the following gradient of solvents A (100 mM  $\text{NH}_4\text{OAc}$ , pH 6.9) and B (isopropanol): equilibrate column and then inject sample at 15% B; hold at 15% B for 10 min; increase B from 15% to 30% over 20 min then hold at 30% B for 10 min; increase from 30% to 80% B over 7 min, then hold at 80% B for the duration. The flow rate was 1.5 ml/min. The effluent was monitored by UV at a wavelength of 254 nm. The HPLC fractions were evaporated to dryness using a Savant Speed-Vac system. Residues were redissolved in 50 mM  $\text{NH}_4\text{OAc}$ , pH 7.4, and assayed in triplicate for their ability to affect binding of [ $^3\text{H}$ ]BTX-B in the screening assay. Figure 11 shows an envelope between fractions 8 and 13 that contain components which enhance the binding of BTX-B as expected for known  $\alpha$ -toxins. In addition, however, two regions, fractions 2 and 18-19, show activity consistent with antagonism of  $\alpha$ -toxins leading to a decrease in binding of BTX-B. Of these two components, fraction 2 was relatively weak and has not been further studied. Fraction 18-19, named  $\delta$ -toxin, appears to be quite potent. Figure 12 presents a dose-response curve for the inhibition of  $\alpha$ -toxin-enhanced BTX-B binding by  $\delta$ -toxin (fraction 18) under screening assay conditions. The apparent  $\text{IC}_{50}$  value is 8  $\mu\text{g/ml}$ .

The effects of  $\delta$ -toxin on nerve action potentials and on the functional antagonism of  $\alpha$ -scorpion toxin (toxin V from *L. quinquestratus*) were tested electrophysiologically using the apparatus described under Experimental Methods. Figure 13A shows that  $\delta$ -toxin has no effect on the action potential at concentrations that effectively inhibit  $\alpha$ -toxin enhancement of BTX-B binding, suggesting that this component is non-toxic. If the nerve is first equilibrated with  $\alpha$ -toxin, leading to a reduction in action potential height and broadening of the waveform, subsequent addition of  $\delta$ -toxin does not reverse the  $\alpha$ -toxin effects, presumably because of the slow dissociation rate of  $\alpha$ -toxin from the sodium channel binding site (Figure 13B). However, if  $\delta$ -toxin is allowed to equilibrate with the nerve prior to the addition of  $\alpha$ -toxin, the effects of the  $\alpha$ -toxin are markedly attenuated (Figure 13C).

Analysis fraction 18-19  $\delta$ -toxin by polyacrylamide gel electrophoresis provided evidence that this material was not pure, so further chromatographic purification was investigated. Initially, rechromatography was attempted on the Dynamax C18 column using a shallow gradient from 30% to 80% B. Two major components were evident in the resulting chromatogram as UV absorbing bands (data not shown), but neither of these two major components were active when tested in the screening assay. Recombining these two fractions also did not result in recovery of activity, demonstrating that activity was not due to a synergistic effect. We conclude that under these conditions the  $\delta$ -toxin may have eluted as a broad band such that the concentration in any single fraction was too low to be detected by the screening assay. Since the two bands that were seen account for the major portion of the UV response of fraction 18-19  $\delta$ -toxin, the active component must be a minor constituent and the potency must be significantly higher than the IC<sub>50</sub> value determined above would indicate.

Further purification of  $\delta$ -toxin was attempted using a different column. An Aquapore BU-300 microbore column ( 2 x 100 mm ) ( a C-4 reversed phase column) was equilibrated with solvent A, 0.1% trifluoroacetic acid (TFA) in 10% isopropanol. Following injection of aliquots of fraction 18-19, a variety of gradients with solvent B, 0.1% TFA in 70% isopropanol, were applied at a flow rate of 0.5 ml/min. Optimal separation was observed with the following gradient (increases in %B between time points were linear):



<u>Time (min)</u>	<u>% B</u>
0	2
5	2
10	10
20	10
25	20
45	20
50	40
60	40
65	60
90	60

The resulting chromatogram is shown in Figure 14. The  $\delta$ -toxin activity was recovered in fraction 20 and 21 as indicated in the Figure. The lack of a 1:1 correspondence between the distribution of activity and the profile of UV peaks in this region of the chromatogram suggests that the  $\delta$ -toxin is still not completely pure. When the active fractions were subjected to rechromatography on the same Aquapore BU-300 microbore column, no activity could be recovered. It is possible that, like  $\alpha$ -scorpion toxins, the  $\delta$ -toxin is quite "sticky" and at low concentrations in low ionic strength media it may be lost to absorption on vessel walls.

The data collected to date suggest that  $\delta$ -toxin is a structural homolog of  $\alpha$ -toxins that retains a relatively high affinity interaction at the Na channel  $\alpha$ -toxin site, yet lacks intrinsic efficacy as a modulator of channel function. Confirmation of this hypothesis, however, must await final purification of this interesting antagonist to homogeneity and analysis of its chemical structure. A summary of the purification of  $\delta$ -toxin to date is given in Table III.

## CONCLUSIONS

The principles which guided development of a rapid screening mechanism for the detection of compounds active at the voltage-sensitive sodium channel were two-fold. 1) The assay should be able to easily accommodate the screening of large numbers of samples as might arise, for example, in the testing of fractions of complex mixtures derived from natural products, from the production of families of monoclonal antibodies, or from synthetic programs in which numerous compounds may be generated. 2) Since the sodium channel exhibits numerous distinct binding domains, many of which are allosterically interrelated, the assay should be general in its ability to detect compounds active at any of these sites in a single procedure.

The first criterion is met by the characteristics of an *in vitro* radioligand binding assay in which a radiolabeled probe is incubated with a suitable sodium channel-containing tissue preparation under control conditions and, in parallel, in the presence of test agents for 45-60 min, simultaneously filtered and washed on a multisample manifold, and analyzed following automated liquid scintillation counting. Synaptoneurosomal preparations of rat brain cerebral cortex offer a significant advantage as a suitable tissue preparation and source of sodium channels, since they are similar to the more traditional synaptosome preparation with respect to sodium channel ligand binding but are much easier to prepare. The utility of this preparation has been extended further by defining conditions under which the synaptoneurosomes could be stored (-70°C) without loss of integrity *vis a vis* sodium channel ligand binding. Using previously prepared synaptoneurosomes that are retrieved from frozen storage, the time required from set-up of the assay to initiation of counting is reduced to only about 2 hr. Thirty samples can routinely be handled in a single assay and be filtered simultaneously with the aid of a manifold. Thus, if each test agent is screened in triplicate, tens of samples could easily be tested in a single day, demonstrating the efficiency of the assay.

The selection of [<sup>3</sup>H]BTX-B as a reporter ligand addresses the second principle by ensuring a large degree of generality in the assay. As demonstrated by the "validation" tests of the procedure, the binding of BTX-B sensitively reports on interactions of ligands at a minimum of five different sodium channel sites. It is interesting to note that several compounds exhibiting structural analogy with active compounds are without effect on [<sup>3</sup>H]BTX-B binding under standard screening assay conditions, suggesting that non-specific mechanism do not account for a positive response. Thus, the catchment area of the

screening assay is quite broad and appears to be specific for the sodium channel. In return for this broad catchment feature, however, it should be noted that the assay is sensitive to potential neurotoxins as well as to potential antitoxins or therapeutic agents. In other words, the assay serves only to identify compounds active at the sodium channel. Once identified, their therapeutic potential and mechanism of action must be tested more directly with other techniques. In our work we have relied on separate ligand binding assays of [ $^3\text{H}$ ]STX and electrophysiological measures to address these aspects. On the other hand, the screening assay does offer some other advantages. Compounds that, by themselves, have little or no effect on sodium channel function yet bind with relatively high affinity to a particular sodium channel site represent one class of potential therapeutic agents since they might be efficacious in antagonizing the actions of a neurotoxin. While it could be difficult to identify such compounds directly by electrophysiological techniques, they would be found using the [ $^3\text{H}$ ]BTX-B screening assay. In summary, we have described a sensitive, simple and rapid screening assay of broad scope which can be of advantage in the identification of potentially therapeutic compounds active at the mammalian voltage-sensitive sodium channel when used with an appreciation of its strengths and weaknesses.

Several examples of potential therapeutic agents for sodium channel poisoning have now been identified in the course of application of the screening assay. Of these, several are small molecular weight compounds that were predicted from previous studies to have therapeutic potential, including the hexahydropyrimidine derivative representing a TTX part-structure (HM-197) and two non-toxic pyrethroid insecticides (CGA-98496 and CGA-98479). Each of these gave a positive response in the screening assay. HM-197 has been studied further by competition binding experiments with [ $^3\text{H}$ ]STX, yielding the unexpected result that effects of HM-197 are not mediated through the TTX/STX sodium channel binding site. Additional binding studies and electrophysiological measurements also appear to eliminate the BTX and local anesthetic domains as HM-197 binding sites. Thus, the binding site and mechanism of action of HM-197 remain unknown, but the protective effect against veratridine or BTX-induced has been demonstrated by concentrations of the compound that have no apparent effects on the action potential. HM-197 remains an interesting and viable therapeutic candidate compound and, in view of its simple structure, future studies of synthetic derivatives may be warranted.

Another line of inquiry that has made substantial use of the screening assay involves the search for potential therapeutic agents directed at scorpion polypeptide sodium channel neurotoxins. Owing to the presence in scorpion venoms of multiple homologous

polypeptide neurotoxins, we hypothesized that there might be homologs present at low concentrations whose sequence rendered them non-toxic without affecting binding affinity, thus providing potential antitoxins. At least two such species have now been identified in the venom of *Leiurus quinquestriatus* by HPLC fractionation and detection with the screening assay. The most potent of these, named  $\partial$ -toxin, has received further study. At concentrations exhibiting activity in the screening assay,  $\partial$ -toxin has no effects on the compound action potential of rat phrenic nerve while inhibiting the action of  $\alpha$ -scorpion toxins.

Two other approaches to scorpion toxin agents were based on the interesting theory that sequence homologies may exist between protein or peptide ligands and their proteinaceous targets. Two separate, homologous regions of the sodium channel protein and  $\alpha$ -scorpion toxins from *L. quinquestriatus* and *A. australis* (Hector), respectively, were in fact identified. Monoclonal antibodies against a synthetic sodium channel peptide representing the former homology have been found to cross react with several components of *L. quinquestriatus* scorpion venom. When a panel of the monoclonal antibodies were probed with the screening assay, weak inhibitory effects were indicated for several. These initial findings should be followed up with additional studies using higher concentrations of the antibodies. Another interesting possibility has been investigated in a preliminary fashion, albeit with negative results. An appropriate synthetic sodium channel peptide could in theory provide a soluble scorpion toxin "pseudoreceptor" that could essentially scavenge the toxin and prevent its interaction with the sodium channel. The strategy of developing synthetic pseudoreceptors based on the natural toxin binding site has obvious potential. Traditionally, one would take the direct approach of characterizing the structure of the natural binding site at the molecular level in order to provide a basis from which to develop pseudoreceptors. For the sodium channel there is very little data concerning a molecular description of the various neurotoxin binding domains. We were therefore quite interested in the potential "short-cut" embodied in the hypothesis that sequence homologies may exist between polypeptide ligands and their proteinaceous receptors and serve to identify receptor sequences that can be synthesized and tested for pseudoreceptor activity. However, when a synthetic sodium channel peptide with sequence homology to *A. australis* (Hector)  $\alpha$ -toxin was tested in the screening assay, no robust effects were found. Although these negative results provide no support for the theory stated above, it is perhaps too early to discard it out of hand. The homologous relationship between portions of the sodium channel and these polypeptide toxins is striking and demands a more satisfactory explanation than serendipity.

#### PERSONNEL ENGAGED IN THIS PROJECT

Dr. George B. Brown, Principal Investigator

Dr. Ronald J. Bradley, Investigator

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#### PUBLICATIONS AND ABSTRACTS RESULTING FROM THIS PROJECT

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Compound	Effect	Reference
$\alpha$ -Scorpion toxin, sea anemone toxin	Increase affinity	(11)
Local anesthetics	Decrease affinity	(12,13)
Diphenylhydantoin and carbamazepine (anticonvulsants)	Decrease affinity	(14)
$\alpha$ -Cyano pyrethroid insecticides	Increase affinity	(15)
TTX and STX	Decrease affinity	(16)
Ciguatoxin	Increase affinity (?)	(17)
Ptychodiscus brevis toxin	Increase affinity	(18,19)

Table I. Allosteric Modifiers of BTX-B Binding



Compound	K <sub>0.5</sub>	Predicted Effect	Observed Effect
BTX-B	300 nM	Inhibit	Inhibit
TTX	20 nM	Inhibit	Inhibit
ScTX	2 µg/ml	Enhance	Enhance
Deltamethrin	1 µM	Enhance	Enhance
CGA-98496	1 µM	Inhibit	Inhibit
CGA-98479	3 µM	Inhibit	Inhibit
HM-197	35 µM	Inhibit	Inhibit
Benzimidazole	>600 µM	?	None
2-β-aminoethyl-benzimidazole	>600 µM	?	None
Nesacaine	15 µM	Inhibit	Inhibit
THP	>600 µM	?	None
THP-OH	>600 µM	?	None

Table II. Effects of ligands on [<sup>3</sup>H]BTX-B binding under standard screening assay conditions.

The compounds listed above were included at varying concentrations in the standard screening assay and the effects on the specific binding of [<sup>3</sup>H]BTX-B at each concentration was recorded as a percentage of control binding without added test agent. The resulting dose response curves were used to determine the K<sub>0.5</sub> values, i.e. that concentration of test agent that produced a half-maximal effect. ScTX refers to the venom of *L. quinquestratus*. CGA-98496 and CGA-98479 are non-toxic stereoisomers of the pyrethroid insecticide cypermethrin. TPH and TPH-OH are tetrahydropyrimidine derivatives supplied by Dr. B. J. Gabrielsen, Fort Detrick.

Material	$\delta$ -Toxin Specific Activity IC <sub>50</sub>	$\mu$ g Protein	Fold Purification
whole venom	-	1000 (venom weight)	-
C18 fraction 18	8 $\mu$ g/ml	14	70
BU300 fraction 20	0.6 $\mu$ g/ml <sup>1</sup>	1.8	555
Repeat BU300	?? <sup>2</sup>	< 0.1	-

<sup>1</sup> Compare  $\alpha$ -toxin V EC<sub>50</sub> = 0.035  $\mu$ g/ml.

<sup>2</sup> Rechromatography of fraction 20 (100  $\mu$ g) over the same Aquapore BU300 column and under identical conditions resulted in failure to recover any activity, suggesting that the highly purified material, similar to  $\alpha$ -toxins, is quite "sticky" and may be lost by adsorption to vessel walls, columns, etc.

**TABLE III. OVERVIEW OF DELTA-TOXIN PURIFICATION**

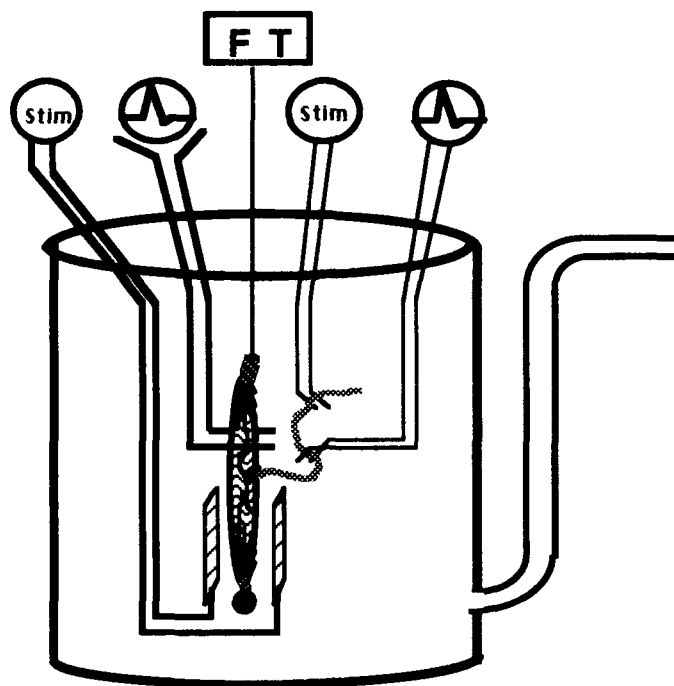


Figure 1. Nerve-muscle chamber for electrophysiological testing

A freshly-dissected nerve-muscle preparation (typically, rat phrenic nerve-diaphragm) is positioned in the apparatus so that the lower portion of the muscle is suspended between two plate electrodes which are used for direct stimulation of the muscle itself. The nerve is passed along two sets of electrodes, the distal pair being used for stimulation and the proximal pair for recording of the nerve compound action potential. Muscle compound action potentials elicited either through stimulation of the nerve or direct stimulation of the muscle with the plate electrodes are recorded with the aid of a third pair of electrodes positioned in the upper half of the muscle. In addition, the muscle is connected at the upper end to a force transducer (FT) for measurement of tension development. The entire assembly is maintained in a beaker containing a buffer (nominally Tyrode's solution) which can be continually bubbled with  $O_2/CO_2$  if required, and to which compounds under investigation can be added. Temperature control is achieved with a thermostatted circulating water bath surrounding the inner beaker. During measurement periods, buffer is withdrawn with a syringe *via* the outlet at the bottom of the beaker until the liquid level is just at the top of the plate electrodes. (The same mechanism may be used to completely withdraw the test solution and introduce fresh buffer to observe the wash-out of effects.) Responses to test stimulations may be recorded on a storage oscilloscope and photographed with a Polaroid camera to produce a permanent record.

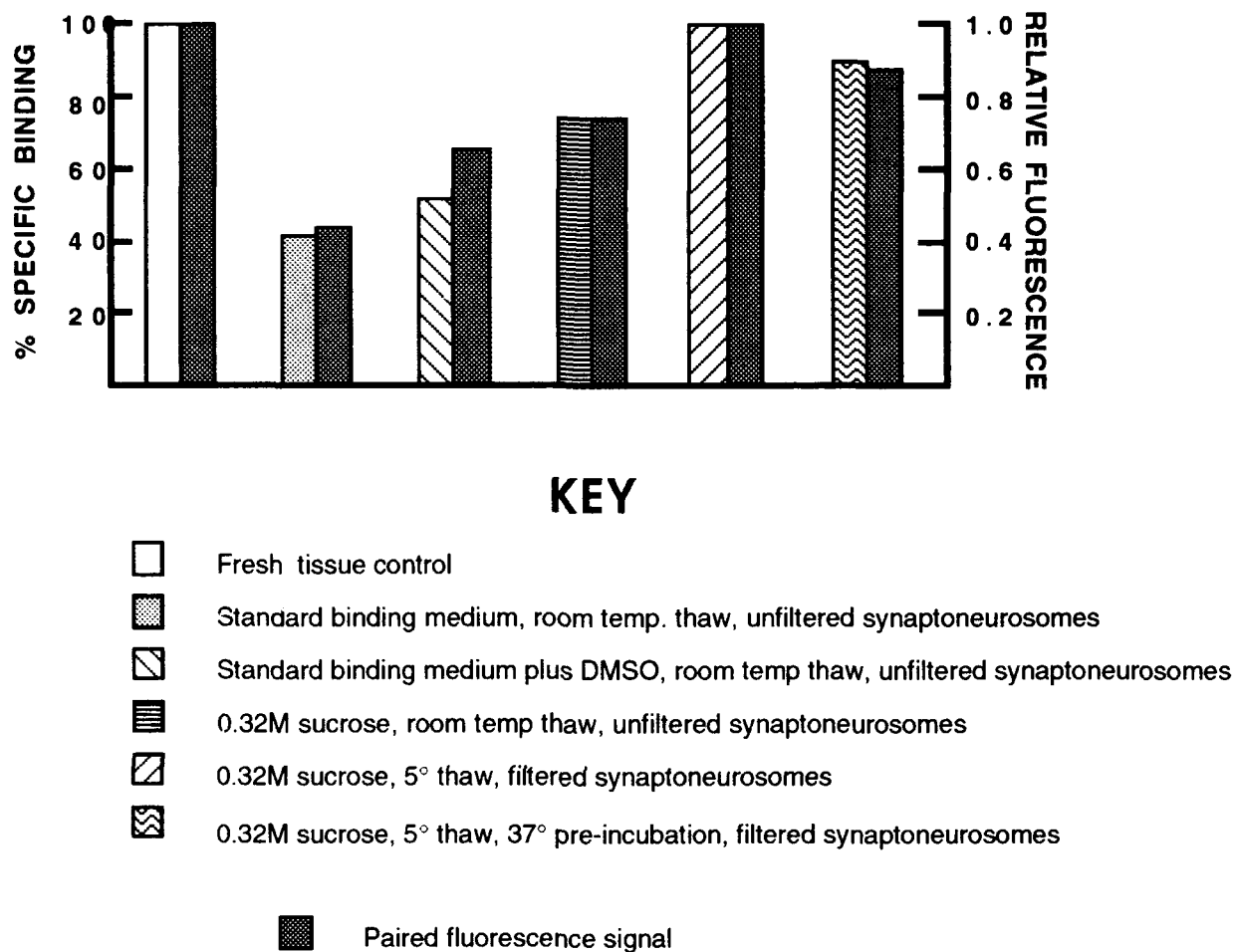


Figure 2. Conditions affecting stability of synaptoneurosomes to frozen storage at  $-70^{\circ}\text{C}$ .

Filtered or unfiltered synaptoneurosomes prepared in the buffers indicated above were stored for three weeks at  $-70^{\circ}\text{C}$ , thawed as indicated and used for determinations of membrane potential and amount of specific BTX-B binding relative to that in freshly prepared synaptoneurosomes. Standard incubation medium refers to HEPES buffer, and standard incubation medium plus DMSO refers to HEPES buffer made 12% in dimethylsulfoxide. Following thawing, the tissue was washed and collected by centrifugation and resuspended in HEPES buffer for the assays as described under Experimental Procedures. Specific BTX-B binding was measured in the presence of a saturating concentration of scorpion toxin. Binding data are reported as the mean of triplicate determinations, whereas the fluorescence measurements are the result of a single determination for each condition.

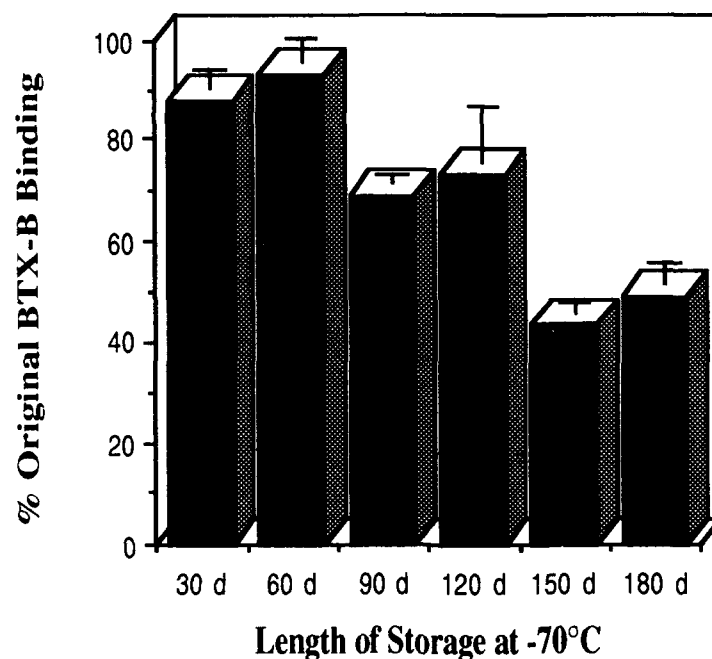


Figure 3. Stability of filtered synaptoneurosomes to extended storage at -70°C.

Synaptoneurosomes were prepared as indicated in Figure 3 and stored in aliquots at -70°C for the periods of time indicated. At monthly intervals, an aliquot was removed and specific binding of BTX-B was measured under standard screening assay conditions. The data are the means  $\pm$  S.D. of triplicate determinations, expressed as percent specific binding normalized to specific BTX-B binding in the freshly-prepared synaptoneurosomes prior to frozen storage.

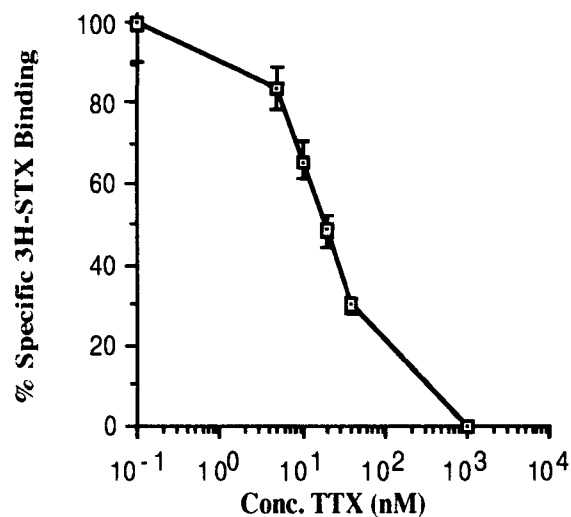


Figure 4. TTX inhibition of [ $^3\text{H}$ ]STX binding in frozen synaptoneurosomes

Synaptoneurosomes were thawed at  $5^\circ\text{C}$  after three months of storage in isotonic sucrose buffer at  $-70^\circ\text{C}$  and used in the assay described in the text to measure the inhibition of specific [ $^3\text{H}$ ]STX binding by TTX. The concentration of [ $^3\text{H}$ ]STX was held constant at 1 nM and the concentration of unlabeled TTX varied between 1-1000 nM. Specific binding at each concentration of TTX is expressed as a percentage of specific binding measured in the absence of TTX. The approximate equilibrium dissociation constant for TTX binding determined from this graph is 17 nM. The data points are the means  $\pm$  S.D. of triplicate determinations.

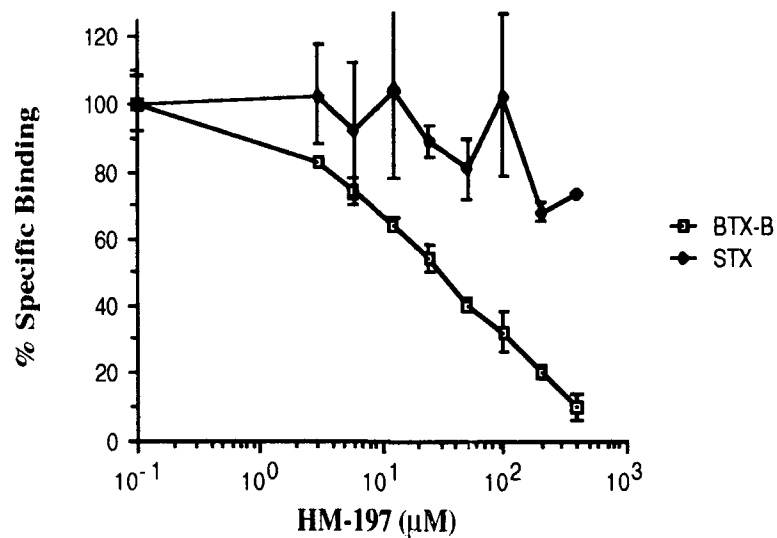


Figure 5. Inhibition of [<sup>3</sup>H]BTX-B and [<sup>3</sup>H]STX binding by HM-197

Binding of [<sup>3</sup>H]BTX-B in the presence of a saturating concentration of scorpion toxin and of [<sup>3</sup>H]STX in the absence of other toxins to freshly prepared synaptoneurosomes was measured as a function of concentration of HM-197. The concentration of labeled BTX-B and STX was held constant at 9 nM and 1 nM, respectively. Data are expressed as a percentage of control binding in the absence of HM-197, and are the means  $\pm$  S.D. of triplicate determinations in a single experiment.

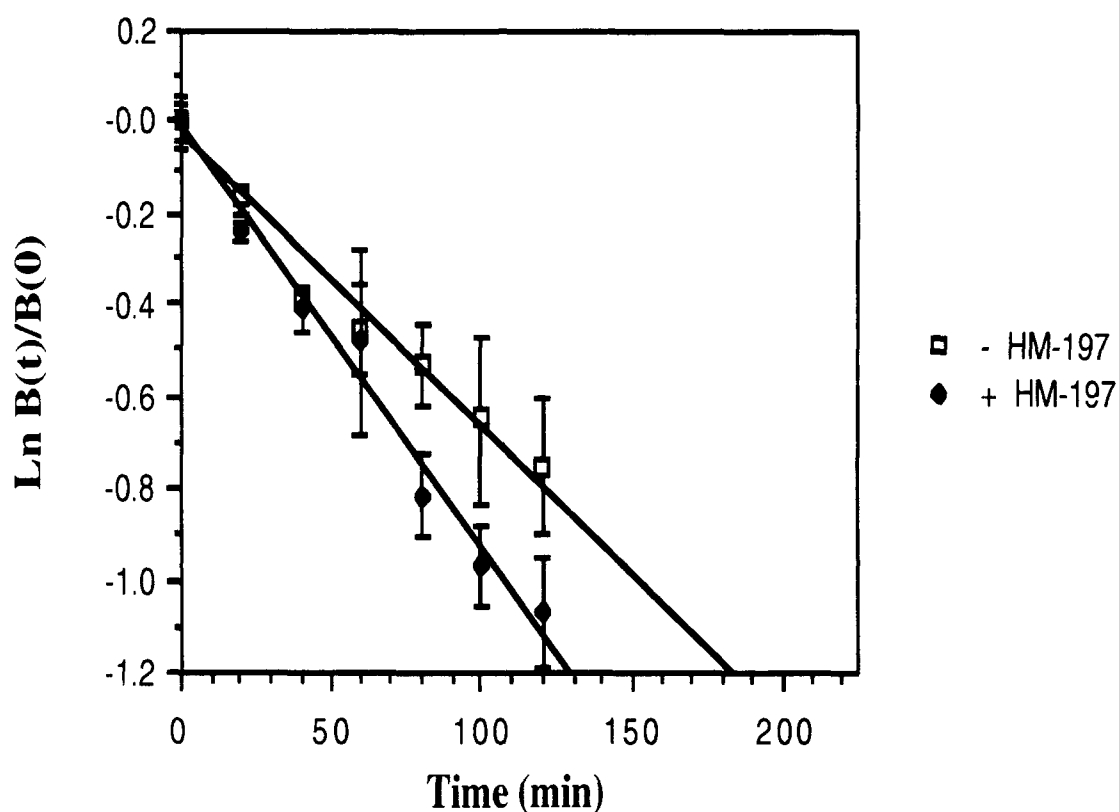
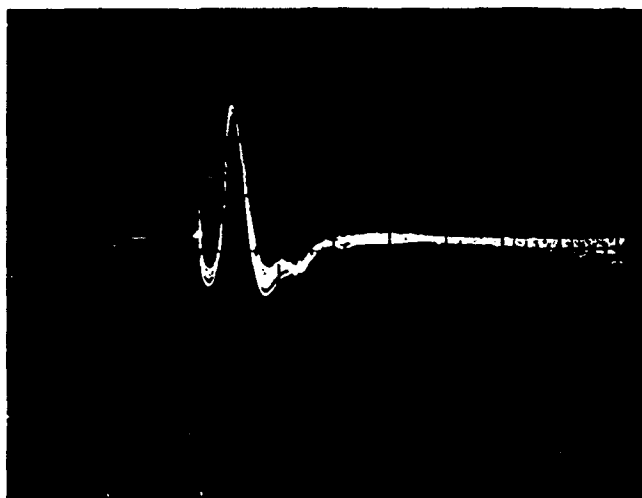


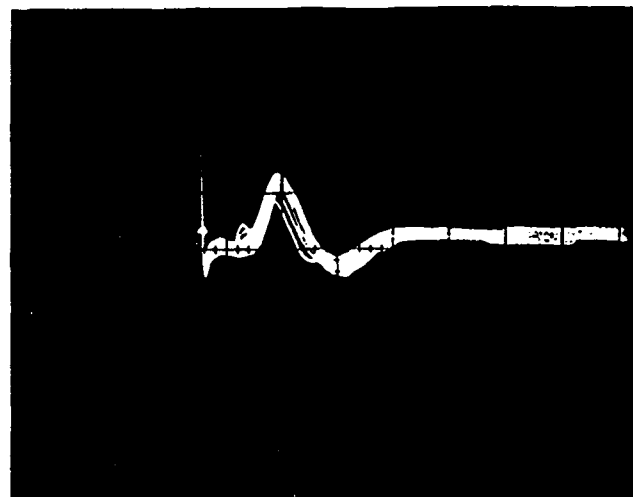
Figure 6. Effect of HM-197 on the dissociation rate for BTX-B binding.

Rat brain synaptoneurosomes were equilibrated with 9 nM [ $^3\text{H}$ ]BTX-B and a saturating concentration of *L. quinquestriatus* scorpion venom (12.5  $\mu\text{g/ml}$ ) for 1 hr, with or without 50  $\mu\text{M}$  HM-197. Excess veratridine (300  $\mu\text{M}$ ) was then added at time 0 and specific binding remaining at the indicated times (t) was measured by filtration assay. The natural logarithm of the specific binding at each time point, B(t), divided by the specific binding at time 0, B(0), is plotted as a function of time. The data are the means  $\pm$  S.D. of triplicate determinations, and the straight lines were fit to the data using linear regression analysis.

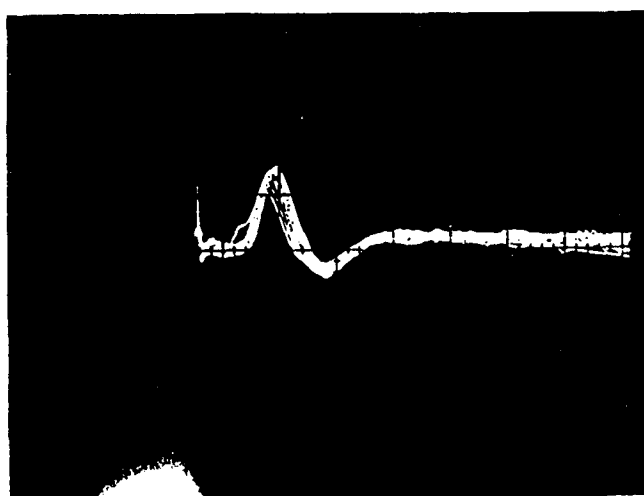




A



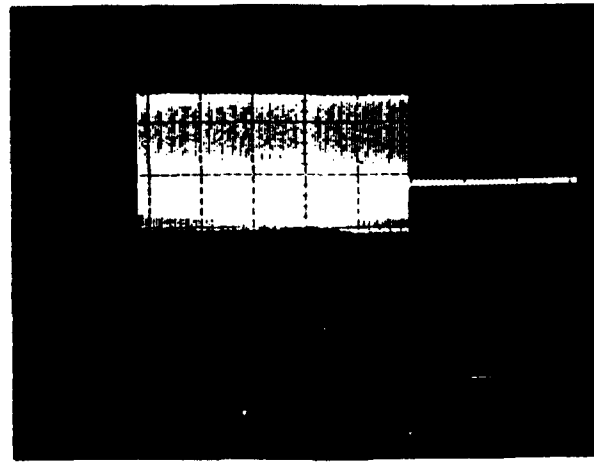
B



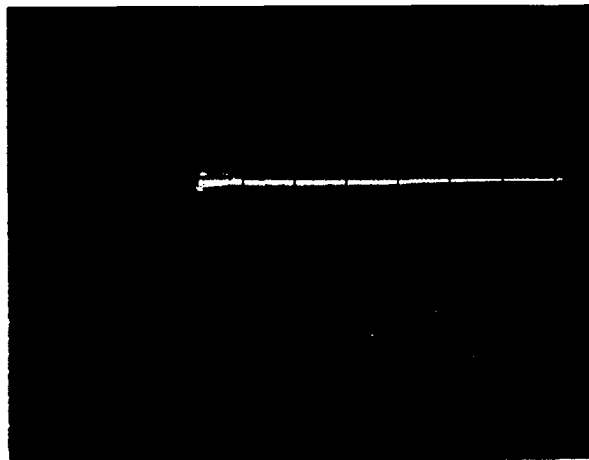
C

Figure 7. Effect of HM-197 on nesacaine-induced block of rat phrenic nerve compound action potential.

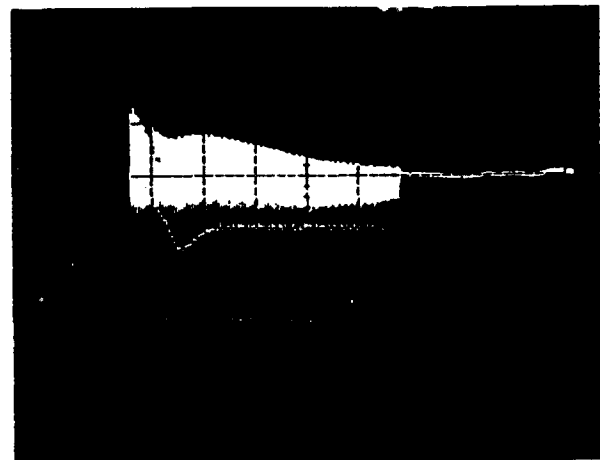
Panel A. Control nerve stimulated supramaximally at 100 Hz. 100 compound action potentials are superposed. Panel B. The nerve was equilibrated with 150  $\mu$ M nesacaine at 30 °C for 30 min prior to stimulation for 1 sec at 100 Hz as in the control. Note the decreased size in the amplitude of the compound action potential and the slight decrementing response indicating a use-dependent effect. Panel C. The nerve was pre-equilibrated with 100  $\mu$ M HM-197 followed by 150  $\mu$ M nesacaine. After 30 min the nerve was stimulated as before. Note the lack of effect of pre-equilibration with HM-197.



8A



8B



8C

Figure 8. Protection against veratridine-induced depolarization of rat phrenic nerve by HM-197

A rat phrenic nerve-diaphragm preparation was mounted in the chamber depicted in Figure 7 and incubated in Tyrode's solution for 60 min in the absence of any effectors (control, 8A), in the presence of 7  $\mu$ M veratridine (8B), or in the presence of 7  $\mu$ M veratridine and 50  $\mu$ M HM-197 added simultaneously (8C). Following this incubation period, the response of the nerve to a train of supramaximal stimuli (100 Hz, 0.5 msec stimulus duration) was recorded on a storage oscilloscope and photographed with a Polaroid camera. Calibration bars: 2 mV  $\times$  0.2 sec.

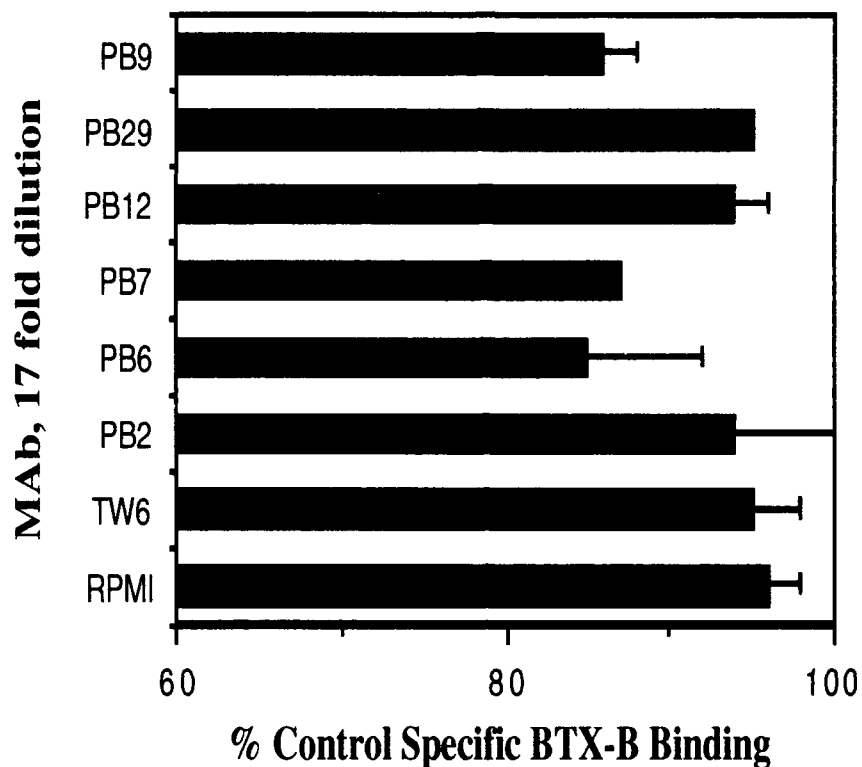


Figure 9. The effects of PB series monoclonal antibodies on BTX-B binding.

Using standard screening assay conditions, the effects of the indicated monoclonal antibodies on specific binding of BTX-B was evaluated. Antibodies were added to the assay tubes as solutions in RPMI cell culture medium. Twenty  $\mu\text{L}$  were diluted into a total assay volume of 338  $\mu\text{L}$ , yielding a dilution factor of 17 for the antibody titer. The results are expressed as a percentage of specific BTX-B binding in control tubes containing no added antibodies. TW6 is a monoclonal antibody raised against an irrelevant antigen and has been used as a control unrelated to the PB series. RPMI refers to the addition of 20  $\mu\text{L}$  of RPMI medium containing no antibodies. The data are expressed as the mean  $\pm$  S.D. of triplicate determinations. The standard error for PB7 and PB29 was 1% and was too small to be visualized upon initial computerized production of the graph.

K P P D C D - P N K V N P G S S V K G D C G N P S V G I F F F  
 V P P - C D G L C K K N G G S S G S C S F L V P S - G L A C W

Figure 10. Amino acid sequence of synthetic sodium channel peptide NAaHI and homology with an alpha-scorpion toxin. Standard one-letter amino acid notations describe the sequence of NAaHI on the top line corresponding to amino acids residues 1776-1805 of the rat brain type I sodium channel sequence. A part structure of the alpha-toxin I from *Androctonus australis* (Hector) is shown on the bottom line, beginning on the left with amino acid # 19 from the amino terminus. Areas of sequence homology between the two are indicated by the boxes.

9/19/88

Dynamax C18 col.

Sample 3/22/88  
Lot 37F-4044

100 $\mu$ l (filter through  
0.45 nylon filter)  
2 mg/100  $\mu$ l

27 fractions  
2.74 min/fraction

UV 254 nm @ 0.2

chart 15 cm/hr

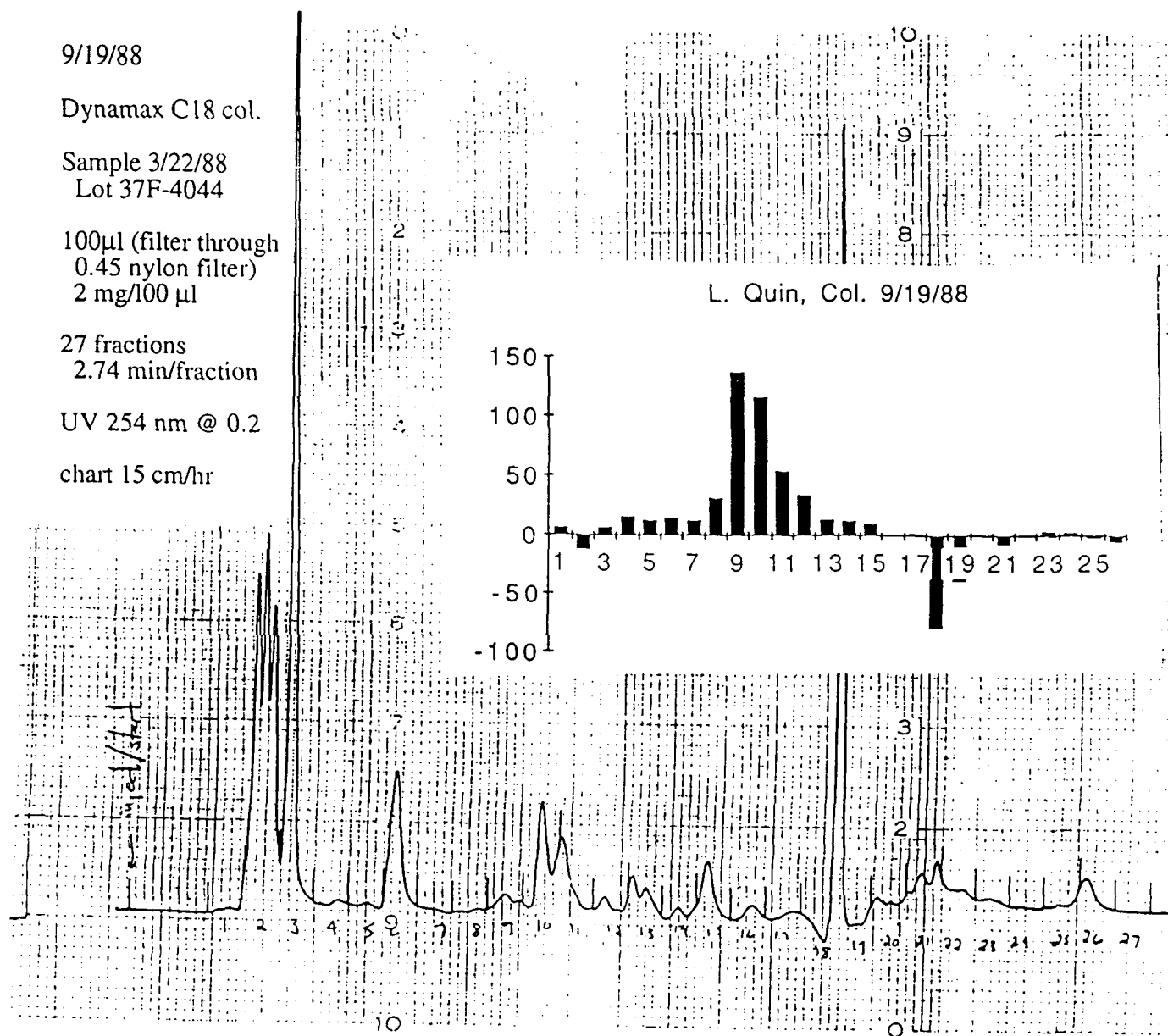


Figure 11. Activities of *L. Quinquestriatus* Venom Fractions. The chromatographic profile for separation of venom components on a C18 reversed phase column is presented. Twenty-seven fractions were collected as indicated, processed, and tested. The insert plots the percentage increase or decrease of control BTX-B binding (ordinate) under the standard screening assay conditions elicited by each fraction (abscissa). Inhibitory activity was routinely recovered in fractions 2 and 18-19. The activity in the latter fractions is the more potent, and has been designated  $\delta$ -toxin.

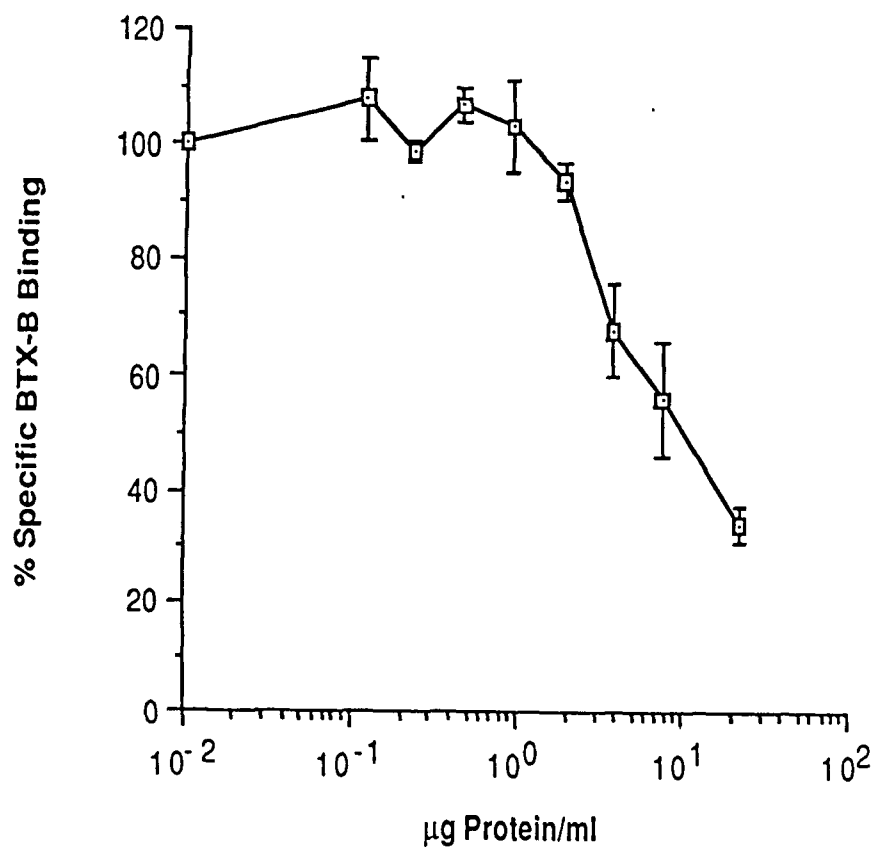
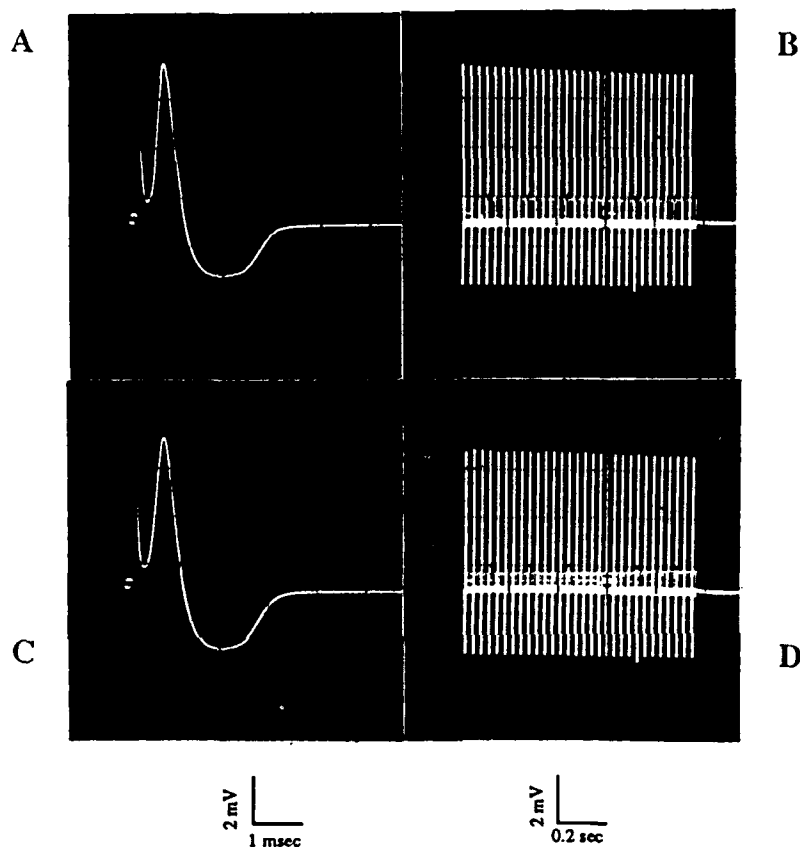


Figure 12. Inhibition of Scorpion Venom-Enhanced BTX-B Binding by  $\delta$ -Toxin. Specific binding of [<sup>3</sup>H]BTX-B to rat brain synaptoneurosomes under standard screening assay conditions was measured as a function of concentration of fraction 18 from C18 HPLC as shown in Figure 11. The apparent IC<sub>50</sub> value for this fractions is 8 µg/ml.

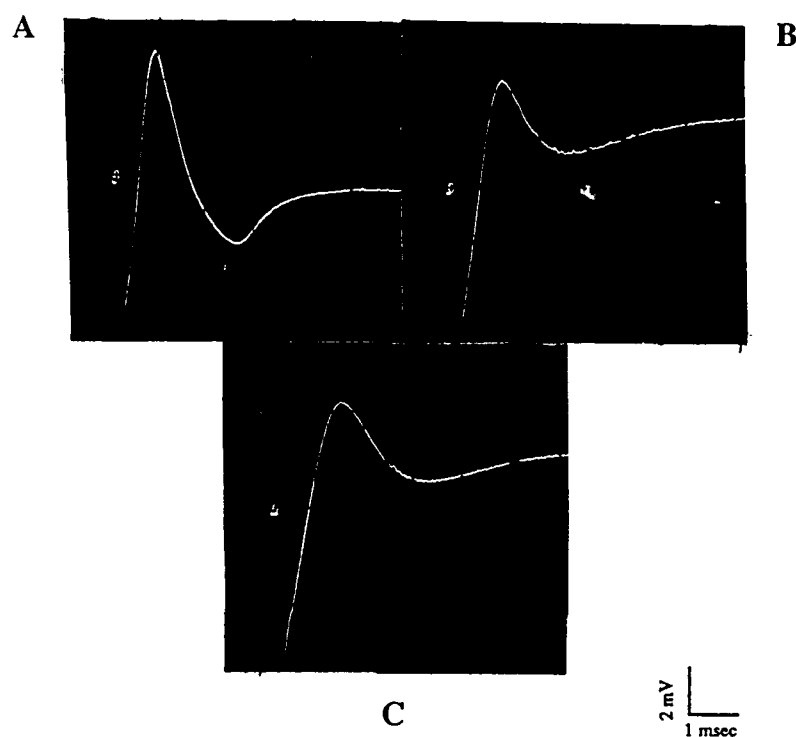


#### EFFECT OF DELTA-TOXIN ON NERVE CAP

A/B = CONTROL

C/D = 40 MIN AFTER 2 MG VENOM EQUIV. TOXIN

Figure 13A.  $\delta$ -Toxin Alone Does Not Affect Rat Phrenic Nerve Compound Action Potential. The active  $\delta$ -toxin fraction from C18 HPLC of *L. quinquestriatus* venom was equilibrated with the nerve at a concentration of 8  $\mu\text{g/ml}$  (corresponding to a whole venom equivalent of 0.28  $\text{mg/ml}$ ) for 40 min. There is no effect on the compound action potential compared to control.



**Effect of Delta-Toxin on Alpha-Toxin Treated Nerve**

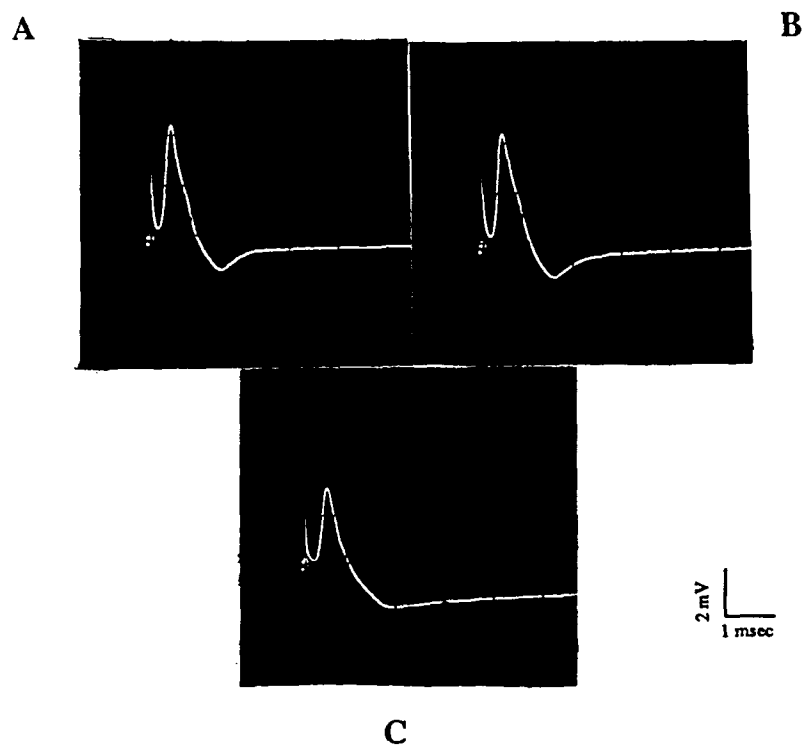
**A = Control**

**B = 40 min after alpha-toxin**

**C = 40 min after addition of delta-toxin**

Figure 13B.  $\delta$ -Toxin Does Not Reverse  $\alpha$ -Toxin Effects on Rat Phrenic Nerve. The marked changes in the compound action potential height and time course induced by  $\alpha$ -scorpion toxin V (*L. Quinquestriatus*) at a concentration of 0.2  $\mu\text{g/ml}$  are not altered or reversed by  $\delta$ -toxin (8  $\mu\text{g/ml}$ ).





# **EFFECT OF DELTA-TOXIN ON ALPHA-TOXIN EFFICACY**

**A = Control**

**B = 40 min after delta toxin**

**C = 40 min after addition of alpha-toxin**

Figure 13C. Pretreatment with  $\delta$ -Toxin Attenuates the Effect of  $\alpha$ -Toxin on Rat Phrenic Nerve.  $\delta$ -Toxin, at a concentration ( $8 \mu\text{g/ml}$ ) that alone has no effect on the compound action potential and that can not reverse  $\alpha$ -toxin-induced changes, markedly attenuates the effects of  $\alpha$ -toxin if allowed to equilibrate with the nerve prior to the addition of  $\alpha$ -toxin.

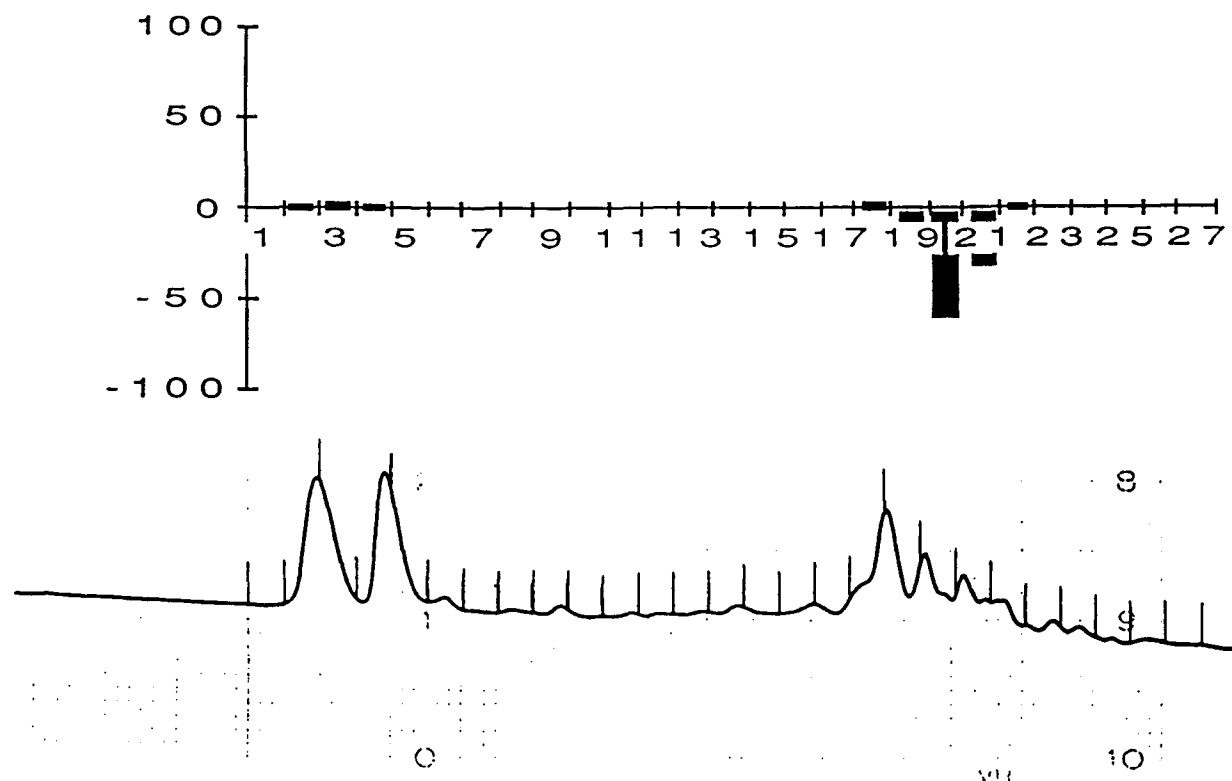


Figure 14. Further Purification of  $\delta$ -Toxin. Fraction 18 containing  $\delta$ -toxin activity derived from chromatography of 10 mg *L. quinquestratus* venom over a Dynamax C18 column was further fractionated on an Aquapore BU300 column as described. Twenty-seven 0.5 ml fractions were collected and 5  $\mu$ l aliquots of selected fractions were tested with the screening assay for inhibition of BTX-B binding. The upper panel shows BTX-B binding in the presence of the various fractions as a percentage of control binding in the absence of any additions. The peak of  $\delta$ -toxin activity was recovered in fraction 20. Note that the chromatographic peaks with the greatest absorbance ( $A_{280}$ ) are devoid of activity in the screening assay

## **APPENDIX**

**A RAPID SCREENING PROCEDURE FOR THE DETECTION  
OF COMPOUNDS  
ACTIVE AT THE VOLTAGE-SENSITIVE SODIUM  
CHANNEL:  
  
A MANUAL**

## I. PREPARATION OF THE TISSUE

### A. Removal of the brain cortex from the animal

1. Anesthetize the animal using anhydrous ether
2. Decapitate the animal immediately posterior to the ears
3. Using scissors cut the skin and peel it back to expose the skull
4. Find the foramen magnum, insert the point of the scissors and cut up the midline of the skull using caution not to cut the brain
5. Pry open the skull
6. Remove the brain and separate the cortex from the remainder of the brain
7. Obtain the weight of the cortex in grams
  - a. animals of the size indicated in appendix A usually have a cortex weight of 0.8 - 1.0 grams
8. Keep the cortex cold by placing on ice

### B. Homogenization of the cortex in Hepes incubation buffer

1. Place the cortex in the small tissue homogenizer (appendix A)
2. Add a volume of ice cold Hepes incubation buffer (appendix B) in milliliters equal to twice the weight of the cortex in grams
3. Use 10 full length strokes with the pestle to homogenize the tissue
4. Empty the homogenizer into the centrifuge tube
5. Rinse the homogenizer with a volume of ice-cold Hepes incubation buffer in milliliters equal to the weight of the cortex plus the volume of buffer added in B.2. and then add the rinse to the centrifuge tube
6. Keep the tissue cold by placing on ice

### C. Centrifugation of the tissue

1. With a tissue volume of 2 - 10 ml per centrifuge tube, centrifuge the tissue at 1000 x gravity for 15 minutes at 4° C
2. Discard the supernatant by decanting and retain the tissue pellet

### D. Resuspension of the tissue pellet

1. Resuspend the pellet in a volume of Hepes incubation buffer in milliliters 20 times the original weight of the cortex
2. Place the tissue and buffer in the large homogenizer and use three full strokes to resuspend

## II. FILTERING OF THE TISSUE

### A. Filtering through the nylon mesh material (appendix A)

1. Use the Buchner style filtering apparatus (appendix A)
2. Cut three layers of nylon mesh and filter the tissue by passive gravity flow through the nylon
3. Collect the filtrate in a flask that is sitting in ice to keep the tissue cold

### B. Next, filtration through Whatman 4 or Whatman 54 paper (appendix A)

1. Use the buchner style filtering apparatus
2. The Whatman 4 paper may need a nylon support due to low wet strength
3. Use the house vacuum or a vacuum pump to collect the filtrate in a flask kept on ice

### C. The final filtration through a Millipore LCWP filter (appendix A)

1. Either the Spectrum or Millipore filtration apparatus may be used
2. Use the house vacuum or a vacuum pump to collect the filtrate in a flask kept on ice
3. Several of these filters used sequentially may be necessary depending upon the volume of tissue filtered, since the filters may become clogged
4. If the glass frit filter apparatus is used, clean the frit in an acid bath

### D. Centrifugation of the tissue

1. With a tissue volume of 10 - 20 milliliters per centrifuge tube, centrifuge the tissue at 1000 x gravity for 30 minutes at 4 °C
2. Discard the supernatant and retain the tissue pellet

## III. RESUSPENSION AND SPECTROPHOTOMETRIC READINGS

### A. Resuspension of the tissue in isotonic sucrose (appendix B)

1. To start, resuspend the pellet using a volume of isotonic sucrose in milliliters equal to five times the original cortex weight

### B. Absorbance reading at 280 nm

1. In a cuvette add 75 microliters of the tissue solution to 1.5 milliliters of water
2. Absorbance at 280 nm should be above 1
3. Adjust the tissue stock solution by adding isotonic sucrose to obtain readings as close to 1 as possible on repeating step 1

#### IV. STORAGE OF THE PREPARED TISSUE

##### A. Aliquot the tissue

1. Place an appropriate amount of thoroughly suspended tissue in the test tubes (appendix A)
2. 2.25 milliliters of tissue per storage tube provides tissue for 12 assay tubes each
3. Cap the test tube (color coding is helpful) and seal with parafilm

##### B. Freezing

1. Freeze the tissue at  $-20^{\circ}\text{C}$  with the tube at an angle
2. Once the tissue is frozen move the tubes to  $-70^{\circ}\text{C}$  for storage up to four months

#### V. THAWING OF THE PREPARED TISSUE

##### A. Thawing of the tissue

1. Thaw the tissue at room temperature (approx. 15-20 min)

##### B. Centrifugation of the tissue

1. Centrifuge the tissue at 1000 x gravity for 20 minutes at  $4^{\circ}\text{C}$
2. Discard the supernatant and retain the tissue pellet

##### C. Resuspension of the tissue pellet

1. Resuspend the tissue in Hepes incubation buffer
2. Use the same amount of Hepes incubation buffer as the volume of tissue placed in the tube for storage

##### D. Incubation of the tissue

1. Incubate the tissue on ice for 15 minutes prior to assay set-up

#### VI. ASSAY PROCEDURES USING THE PREPARED TISSUE

##### A. Set-up of the assay

1. Assay set-up is in triplicate
2. An assay must include one set of triplicates for total binding and one set of triplicates for non-specific binding
  - a. The total binding set uses 150 microliters of tissue + 168 microliters of toxin mix per assay tube (appendix D)
  - b. The non-specific binding set uses the above quantities plus 8 microliters of 10 mM Veratridine per assay tube
3. To test various compounds add an amount under 10 microliters to the 150 microliters tissue + 168 microliters toxin mix to obtain the necessary concentration of the compound in the assay

4. Vortex the samples
  - a. Control tubes should be included that contain the same volume of test compound vehicle
  - b. If test compounds are in organic solvents, they should be added to assay tubes first and allowed to evaporate before adding tissue and toxin cocktail
- B. Incubation of the assay
  1. Incubate the assay for 45 minutes at room temperature
- C. Harvesting of the assay
  1. Prepare the harvester according to the manufacturer's instructions (appendix A)
  2. Use Whatman GF/C filter paper to collect the samples
  3. After the incubation dilute the sample with 3 milliliters of cold Hepes wash buffer (appendix B) to stop the reaction
  4. Harvest the sample
  5. Wash the sample tubes three more times with 3 milliliters of Hepes wash buffer and harvest the washes
  6. The Hepes wash buffer must be cold, 4° C
- D. Counting the samples by liquid scintillation
  1. Place the GF/C filters in liquid scintillation counting vials
  2. Add 5-10 milliliters of counting fluid cocktail to the vials
  3. Count the samples for an appropriate time



## APPENDIX A MATERIALS REQUIRED

### SUPPLIES:

Decapitator	
Homogenizer -----	Tenbroeck 7 ml (Kontes) 20 ml (Thomas)
Ice Bucket	
Centrifuge -----	capable of 1000 x gravity (Sorvall RC-5B)
Appropriate tubes	
Balance	
Graduated cylinders or pipets	
Filter apparatus -----	Spectra-Mesh Buchner style (5.5 cm) Spectrum Microfiltration System (47 mm) or Millipore XX10 047 00 Glass 47 mm filter holder assembly
Nylon Mesh -----	160 micron (Tetko, Inc. Elmsford, New York)
Whatman 4 or 54 -----	5.5 cm circles
Millipore LCWP 047 -----	10 micron filters, 47mm circles
Spectrophotometer	
Cuvettes (quartz)	
Test tube with cap -----	capable of 1000 x g (Sarstedt 55.481) caps (Sarstedt 65.793 colors)
Parafilm	
-20°C and -70°C freezer	
Harvester -----	M-24R or M-30R (Brandel, Gaithersburg, and accessories Maryland)
Microliter pipets -----	1 to 200 microliter range
Pipet tips	
Test tubes -----	Sarstedt 55.475
Whatman GF/C filter paper	
Liquid scintillation counter --	(Beckman 3800)

### ANIMALS:

Charles River Sprague Dawley male rats 180-200g

### CHEMICALS:

Ether, anhydrous  
 Choline Chloride  
 HEPES Buffer  
 Glucose  
 Magnesium Sulfate  
 Potassium Chloride  
 Tris Base  
 Calcium Chloride  
 Sucrose  
 Sodium Phosphate Monobasic  
 Leiurus quinquestriatus venom from North Africa (Sigma V5251) (ScTX)

## APPENDIX B SOLUTIONS AND BUFFERS

### INCUBATION BUFFER

	100 ml	200ml	500ml	
130 mM Choline Chloride	1.8	3.63	9.08	grams
50 mM HEPES	1.19	2.38	5.96	grams
5.5 mM Glucose	0.1	0.2	0.495	grams
0.8 mM MgSO <sub>4</sub>	0.02	0.04	0.0985	grams
5.4 mM KCL	0.04	0.08	0.2015	grams

Bring to appropriate volume with deionized water and pH to 7.4 with Tris Base

May be stored at 4° C for 2 weeks

### WASH BUFFER

	2 liter	4 liter	
163 mM Choline Chloride	45.51	91.0	grams
5 mM HEPES	2.38	4.8	grams
1.8 mM CaCl <sub>2</sub>	0.53	1.06	grams
0.8 mM MgSO <sub>4</sub>	0.39	0.789	grams

Bring to appropriate volume with deionized water and pH to 7.4 with Tris Base

May be stored at 4° C for 2 weeks

### ISOTONIC SUCROSE

10 mM Sodium Potassium Monobasic	137.99mg/100ml
0.32 M Sucrose	11g/100ml

Bring to just under volume and adjust pH to 7.4 with Tris Base or NaOH then bring to volume

Deltamethrin (DM)  
Tetrodotoxin (Sigma T6254) (TTX)  
Batrachotoxinin-A Benzoate tritiated (New England Nuclear) ( $^3\text{H}$ -BTX-B)  
Veratridine (Sigma V5754)  
Research Products International Counting Cocktail 3a70B

APPENDIX C

Sample Form for Tracking Synaptoneurosome Preparation and Usage

FROZEN SYNAPTONEUROSOME PREP

DATE: \_\_\_\_\_

CAP COLOR: \_\_\_\_\_

NUMBER OF VIALS (2.25 ml ): \_\_\_\_\_

ADDITIONAL VIALS \_\_\_\_\_ WITH \_\_\_\_\_ ml

BRAIN WEIGHTS: ( \_\_\_\_\_ )( \_\_\_\_\_ )( \_\_\_\_\_ )( \_\_\_\_\_ )( \_\_\_\_\_ )( \_\_\_\_\_ )

TOTAL BRAIN WEIGHT: \_\_\_\_\_

FINAL DILUTION FACTOR: \_\_\_\_\_

A 280 READINGS: 75 microliter IN 1.5 ml

SLIT WIDTH .5  
STRAY LIGHT NO  
BLANK YES

READING \_\_\_\_\_

ADJUSTMENT FACTOR (IF NECESSARY)

\_\_\_\_\_

TEST ASSAY RESULTS

3H-BTX-B: \_\_\_\_\_

CPM/50: \_\_\_\_\_

NORMAL: \_\_\_\_\_ +V \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

SPECIAL NOTES PERTAINING TO THIS PREP:

## APPENDIX D

### ASSAY VOLUMES

ScTX final concentration 2.5 ug/ml in the assay	stock solution: 2mg/ml in water*
DM final concentration 1 uM in the assay	stock solution: 5mM in methanol
TTX final concentration 25 nM in the assay	stock solution: 100uM in water
3H-BTX-B final concentration 9 nM in the assay	stock solution: 1:10 dilution in methanol of NEN product NET-856

#### TOXIN COCKTAIL

To 2.51 ml of HEPES incubation buffer add:  
for 12 assay tubes

6 ul ScTX stock solution  
0.95 ul DM stock solution  
1.2 ul TTX stock solution

add <sup>3</sup>H-BTX-B (specific activity of 50 Ci/mmol) to obtain 50,000 cpm/50 ul in the toxin cocktail in order to attain a concentration of approximately 9 nM in the assay

Use 168 ul of the toxin cocktail per assay tube + 150 ul of tissue per assay tube

Use 8 ul of 10mM veratridine in methanol per assay tube to obtain non-specific binding

#### \*PREPARATION OF SCTX STOCK SOLUTION

Weigh desired amount of ScTX for a 2 mg/ml solution in deionized water.

Stir the solution at 40°C for 1 hour.

Centrifuge the solution at 100,000 x gravity for 45 minutes.

Decant and save the supernatant.

Store frozen at -20°C in aliquots of a size to prevent repeated thawing and freezing